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(57) Abstract

The present invention relates to a newly identified family of protein serine/threonine kinases which phosphorylate microtubule-associated protein 2 (MAP2). It is based, in part, on the cloning and characterization of novel MAP2 kinases designated extracellular signal-regulated kinase 1, 2, and 3 (ERK1, ERK2, ERK3) which are expressed in the central nervous system, and on the identification of another ERK family member, ERK4, with antisera. The present invention provides fore recombinant nucleic acid molecules and proteins representing members of the MAP2 kinase family, and also for microorganisms, transgenic animals, and cell lines comprising recombinant MAP2 kinase molecules. In additional embodiments of the invention, the present invention provides for methods for assaying cellular factor activity, including, but not limited to, nerve growth factor activity, in which the activation of MAP2 kinase serves as an indicator of cellular factor activity. These methods may be extremely useful in screening compounds for the presence of a desired cellular factor activity. In specific embodiments, compounds which may be useful in the treatment of Alzheimer's disease , peripheral neuropathies, and diabetes may be identified using the methods of the invention.

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A FAMILY OF MAP2 PROTEIN KINASES

1. INTRODUCTION

The present invention relates to a newly identified family of MAP2 protein kinases. It is based, in part, on the cloning and characterization of three MAP2 protein kinases, designated ERK1, ERK2, and ERK3, which are expressed in the central nervous system and elsewhere. The present invention provides for recombinant MAP2 kinase nucleic acids and proteins, cell lines and microorganisms comprising recombinant MAP2 kinase molecules, and bioassay methods for detecting the presence of biologically active compounds which utilize recombinant MAP2 kinase molecules.

2. BACKGROUND OF THE INVENTION

2.1. PROTEIN KINASE CASCADES AND THE REGULATION OF CELL FUNCTION

A cascade of phosphorylation reactions, initiated by a receptor tyrosine kinase, has been proposed as a potential transducing mechanism for growth factor receptors, including the insulin receptor (Cobb and Rosen, 1984, Biochim. Biophys. Acta. 738:1-8; Denton et al., 1984, Biochem. Soc. Trans. 12:768-771). In his review of the role of protein phosphorylation in the normal control of enzyme activity, Cohen (1985, Eur. J. Biochem. 151:439-448) states that amplification and diversity in hormone action are achieved by two principal mechanisms, the reversible phosphorylation of proteins and the formation of "second messengers"; many key regulatory proteins are interconverted between phosphorylated and unphosphorylated forms by cellular protein kinases and certain protein phosphatases.

Some hormones appear to transmit their information to the cell interior by activating transmembrane signalling systems that control production of a relatively small

number of chemical mediators, the "second messengers." These second messengers, in turn, are found to regulate protein kinase and phosphatase activities, thereby altering the phosphorylation states of many intracellular proteins, and consequently controlling the activity of enzymes which are regulated by their degree of phosphorylation (see Figure 1). The receptors for other hormones are themselves protein kinases or interact directly with protein kinases to initiate protein kinase signalling cascades. These series of events are believed to explain the diversity associated with the actions of various hormones (Cohen, 1985, Eur. J. Biochem. 151:439-448; Edelman et al., 1987, Ann. Rev. Biochem. 56:567-613).

Insulin, like most cellular regulators, exerts its effects on many cellular processes through alterations in the phosphorylation state of serine and threonine residues within regulated proteins. Insulin exerts these effects via its receptor, which has intrinsic tyrosine-specific protein kinase activity (Rosen et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:3237-3240; Ebina et al., 1985, Cell 40:747-758). Of note, the proteins encoded by several oncogenes are also protein-tyrosine kinases. For example, $P68^{gag-ros}$, a transmembrane transforming protein, bears many similarities to the insulin receptor, sharing 50% amino acid identity (for discussion, see Boulton et al., 1990, J. Biol. Chem. 265:2713-2719).

Nerve growth factor (NGF), a neurotrophic agent necessary for the development and function of certain central and peripheral nervous system neurons, is also believed to influence cellular functions, at least in part, by altering phosphorylation of intracellular proteins. It has been observed that NGF promotes changes in the phosphorylation of certain cellular proteins (discussed in Volonte et al., 1989, J. Cell. Biol. 109:2395-2403; Aletta et al., 1988, J. Cell. Biol. 106:1573-1581; Halegoua and

Patrick, 1980, Cell 22:571-581; Hama et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:2353-2357; Romano et al., 1987, J. Neurosci, 7:1294-1299). Furthermore, NGF appears to regulate several different protein kinase activities (Blenis and Erikson, 1986, EMBO J. 5:3441-3447; Cremins et al., 1986, J. Cell Biol. 103:887-893; Landreth and Rieser, 1985, J. Cell. Biol. 100:677-683; Levi et al, 1988, Mol. Neurobiol. 2:201-226; Mutoh et al., 1988, J. Biol. Chem. 263:15853-15856; Rowland et al., 1987, J. Biol. Chem. 262:7504-7513). Mutoh et al. (1988, J. Biol. Chem. 263:15853-15856) reports that NGF appears to increase the activities of kinases capable of phosphorylating ribosomal protein S6 (S6 kinases) in the PC12 rat pheochromocytoma cell line, a model system regularly used to study NGF function. Volonte et al. (1989, J. Cell. Biol. 109:2395-2403) states that the differential inhibition of the NGF response by purine analogues in PC12 cells appeared to correlate with the inhibition of PKN, an NGF-regulated serine protein kinase. Additionally, activators of the cyclic AMP dependent protein kinase (PKA) and protein kinase C (PKC) have been reported to mimic some but not all of the cellular responses to NGF (Levi et al., 1988, Mol. Neurobiol. 2:201-226). Miyasaka et al. (1990, J. Biol. Chem. 265:4730-4735) reports that NGF stimulates a protein kinase in PC12 cells that phosphorylates microtubule-associated protein-2. Interestingly, despite the many reports linking NGF with changes in phosphorylation of cellular proteins, analysis of a cDNA sequence encoding a subunit of the NGF receptor which is sufficient for low-affinity binding of ligand has indicated no evidence for a protein-tyrosine kinase domain in the cytoplasmic region of this low affinity receptor (Johnson et al., 1986, Cell 47:545-554).

2.2. MAP2 PROTEIN KINASE

Ribosomal protein S6 is a component of the eukaryotic 40S ribosomal subunit that becomes phosphorylated on multiple serine residues in response to a variety of mitogenic stimuli, including insulin, growth factors and various transforming proteins (for discussion, see Sturgill et al., 1988, Nature 334:715-718). Recently, an activated S6 kinase has been purified and characterized immunologically and molecularly (Ericson and Maller, 1986, J. Biol. Chem. 261:350-355; Ericson et al., 1987, Mol. Cell Biol. 7:3147-3155; Jones et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:377-3381; Gregory et al., 1989, J. Biol. Chem. 264:18397-18401). Reactivation and phosphorylation of the S6 kinase occurs *in vitro* via an insulin-stimulated microtubule-associated protein-2 (MAP2) protein kinase providing further evidence for a protein kinase cascade (Sturgill, 1988, *supra*; Gregory et al., 1989, *supra*). MAP2 kinase has been observed to phosphorylate microtubule-associated protein-2 (MAP2) on both serine and threonine residues (Ray and Sturgill, 1987, Proc. Natl. Acad. Sci. U.S.A. 84:1502-1506; Boulton et al., 1991, Biochem. 30:278-286). These observations suggest that key steps in insulin action involve the sequential activation by phosphorylation of at least two serine/threonine protein kinases (Sturgill et al., 1988, Nature 334:715-718; Gregory et al., 1989, J. Biol. Chem. 264:18397-18401; Ahn et al., 1990, J. Biol. Chem. 265:11495-11501), namely, a MAP2 kinase and an S6 kinase. The MAP2 kinase appears to be activated transiently by insulin prior to S6 kinase activation.

The MAP2 kinase phosphorylates S6 kinase *in vitro* causing an increase in its activity (Gregory et al., 1989, J. Biol. Chem. 264:18397-18401; Sturgill et al., 1988, Nature, 334:715-718); thus, the MAP2 kinase is a likely intermediate in this protein kinase cascade. The finding

that phosphorylation on threonine as well as tyrosine residues is required for MAP2 kinase activity (Anderson et al., 1990, Nature, 343:651-653) suggests that it, like many other proteins, is regulated by multiple phosphorylations. The phosphorylations may be transmitted through one or several signal transduction pathways.

In addition to stimulation by insulin, MAP2 kinase activity can be rapidly increased by a variety of extracellular signals which promote cellular proliferation and/or differentiation. In this regard, MAP2 kinase may be equivalent to pp42 (Cooper and Hunter, 1981, Mol. Cell. Biol. 1:165-178), a protein whose phosphotyrosine content increases following exposure to growth factors and transformation by viruses (Rossamondo et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6940-6943) and activation of the v-ros oncogene (Boulton et al., 1990, J. Biol. Chem. 265:2713-2719). For example, MAP2 kinase activity is stimulated in: terminally differentiated 3T3-L1 adipocytes in response to insulin (Ray and Sturgill, 1987, Proc. Natl. Acad. Sci. U.S.A. 84:1502-1506); in post-mitotic adrenal chromaffin cells in response to signals that induce catecholamine secretion (Ely et al., 1990, J. Cell Biol. 110:731-742); in PC12 cells in response to nerve growth factor-induced neuronal differentiation (Volonte et al., J. Cell Biol. 109:2395-2403; Miyasaka et al. J. Biol. Chem. 265:4730-4735) and in T lymphocytes (Nel et al., 1990, J. Immunol. 114:2683-2689). MAP2 kinase(s) are likely to play important roles in signal transduction in many different pathways and in a wide variety of cell types.

Ray and Sturgill (1988, J. Biol. Chem. 263:12721-12727) describes some chromatographic properties of a MAP2 kinase and reports the biochemical characteristics of the partially purified enzyme. MAP2 kinase was observed to have an affinity for hydrophobic chromatography matrices. The molecular weight of the partially purified enzyme was

observed to be 35,000 by gel filtration chromatography and 37,000 by glycerol gradient centrifugation. MAP2 kinase activity of chromatographic fractions was found to correlate with the presence of a 40 kDa phosphoprotein detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). MAP2 kinase was observed to have a Km of 7 μM for ATP, and did not appear to utilize GTP. It has been observed that MAP2 kinase requires phosphorylation on tyrosine as well as serine/threonine residues for activity. Ray and Sturgill (supra) cite several problems encountered in the purification of MAP2 kinase, most notably, the presence of contaminating kinases observed to phosphorylate MAP2 *in vitro*. In addition, only very small amounts of only partially purified protein were available following chromatographic preparation. As discussed supra, Rossomando et al. (1989, Proc. Natl. Acad. Sci. U.S.A. 86:6940-6943) have suggested that MAP2 kinase may be a tyrosine-phosphorylated form of pp42, a low abundance 42-kDa protein which becomes transiently phosphorylated on tyrosine after cell stimulation with a variety of mitogens. Rossomondo et al. (supra) observed that phosphorylation of pp42 and activation of MAP2 kinase occur in response to the same mitogens, that the two proteins comigrate on two dimensional polyacrylamide gels and have similar peptide maps, and that the two proteins copurify during sequential purification on anion-exchange, hydrophobic interaction and gel-filtration media.

3. SUMMARY OF THE INVENTION

The present invention relates to a newly identified family of protein serine/threonine kinases which phosphorylate microtubule-associated protein 2 (MAP2). It is based, in part, on the cloning and characterization of novel MAP2 kinases designated extracellular signal-regulated kinase 1, 2, and 3 (ERK1, ERK2, ERK3) which are

expressed in the central nervous system, and on the identification of another ERK family member, ERK4, with antisera. Accordingly, the term "MAP2 kinase" as used herein shall mean a member of the MAP2 family of kinases, including but not limited to ERK1, ERK2, and ERK3.

The present invention provides for recombinant nucleic acid molecules and proteins representing members of the MAP2 kinase family, and also for microorganisms, transgenic animals, and cell lines comprising recombinant MAP2 kinase molecules. In additional embodiments of the invention, the present invention provides for methods for assaying cellular factor activity, including, but not limited to, nerve growth factor activity, in which the activation of MAP2 kinase serves as an indicator of cellular factor activity. These methods may be extremely useful in screening compounds for the presence of a desired cellular factor activity. In specific embodiments, compounds which may be useful in the treatment of Alzheimer's disease, peripheral neuropathies, and diabetes may be identified using the methods of the invention.

4. DESCRIPTION OF THE FIGURES

FIGURE 1. Schematic diagram of the relationship between hormone binding to a cellular receptor and consequent changes in the phosphorylation of proteins.

FIGURE 2. A. SDS-PAGE analysis of final Q-Sepharose #2 fractions isolated from NGF-treated or control PC12 cells. Aliquots of fractions obtained from the final purification column (Q-Sepharose#2) were concentrated and then analyzed via 15% SDS-PAGE. Note that fractions containing the most MAP2 kinase activity contained a prominent band (arrow) with a molecular weight of approximately 43,000 kD, as described for the insulin-stimulated MAP2 kinase BSA. Ovalbumin and cytochrome C are presented as size standards.

B. The complete nucleotide sequence (SEQ ID NO:1) of the ERK1 cDNA and its predicted protein product (SEQ ID NO:2). Asterisks denote the residues most conserved among all protein kinases. The sequences of the nine tryptic peptides that were sequenced are underlined. All residues precisely determined by the amino acid sequencing matched the cDNA encoded protein sequence; questionable residues were verified from the cDNA encoded protein sequence. The fourth and seventh peptides indicated represented the minor peptide components described in the text.

FIGURE 3. Nucleotide and predicted protein sequences of ERK2 and ERK3 cDNAs and ERK1Ψ pseudogene. Initiation and termination codons are boxed; approximate locations of protein kinase subdomains indicated by roman numerals; asterisks denote residues most conserved among all protein kinases (Hanks et al., 1988, Science 241:42-52); and pound signs denote which of these residues are not conserved in the indicated sequences.

A. Nucleotide (SEQ ID NO:3) and predicted protein (SEQ ID NO:4) sequence of one of the two ERK2 cDNA clones; protein coding region of the other ERK2 cDNA matches exactly, although sequences in the flanking regions diverged.

B. Complete nucleotide (SEQ ID NO:5) and predicted protein (SEQ ID NO:6) sequence of one of two ERK3 cDNA clones analyzed; sequence of the other ERK3 cDNA matches exactly although there were differences in the amounts of flanking sequence.

C. Alignment of partial sequence of ERK1Ψ with the ERK1 nucleotide sequences; only amino acid differences (including the premature termination codon of ERK1Ψ, which is boxed) from the ERK1 protein sequence are

indicated. Dashes indicate deletions in both the nucleotide and amino acid sequences.

FIGURE 4. Comparison of ERKs with FUS3, KSS1 and human cdc2 protein sequences.

A. Computer-generated alignments (MacVector Computer Analysis Software, International Biotechnologies, Inc., New Haven, CT) were visually optimized. Roman numerals indicate subdomains conserved in protein kinases (Hanks et al., 1988, Science 241:42-52). Dots indicate identity to ERK1 sequence, dashes indicate spaces introduced to improve sequence alignments.

B. Percent identities between the sequences aligned in A, determined over the length of the cdc2+sequence; mismatches, insertions and deletions between two sequences all weighted equally.

FIGURE 5. Use of ERK1-, ERK2- and ERK3-specific probes provides evidence for additional ERK genes.

A. Specificity of each of the ERK probes (described in Materials and Methods) was demonstrated by hybridizing three triplicate Southern blots, each with linearized plasmids containing the ERK1, ERK2 and ERK3 cDNA inserts (as marked for each lane), with each of the ERK probes as indicated below the blots.

B. Probing of Southern blots containing EcoR1-digested rat and human genomic DNA with each of the ERK-specific probes; sizes of DNA fragments indicted in kilobases.

C. Probing of Southern blots containing rat genomic DNA digested with Bgl2, BamH1 and Hind3 with each of the ERK-specific probes; sizes of DNA fragments indicated in kilobases.

FIGURE 6. Independent regulation of ERK transcripts in tissues, developmentally, in cultured astroglia and in the P19 embryocarcinoma cell line.

A. Distinct patterns of expression for each of the ERKs within adult nervous system, in adult peripheral

tissues, and in placenta. Specific probes for each of the ERKs (see Figure 3) were hybridized to Northern blots containing 10 μg of RNA from the indicated adult tissues and brain regions. ADR, adrenal; RET, retina; SC.N., sciatic nerve; S.C., spinal cord; A.BR, adult brain; CBL, cerebellum; HBR, hindbrain; MBR, midbrain, DIEN, diencephalon; STR, striatum; HIP, hippocampus; CTX, neocortex; OLF, olfactory bulb; SKIN, skin; HRT, heart; MUS, muscle; LUNG, lung; INT, intestine; KID, kidney; LIV, liver; SPL, spleen; THY, thymus; PLAC, placenta.

B. ERK transcripts are developmentally regulated within the nervous system and in peripheral tissues. Ten μg of total RNA isolated from the indicated developmental stages (E: embryonic day; P:post-natal day; AD: adult) of rat brain, spinal cord, hippocampus (HIPP), liver and heart were compared for hybridization to each of the ERK-specific probes.

C. ERK2 and ERK3 transcripts expressed at low levels in cultured astroglia. Ten micrograms of total RNA from adult rat brain (BRN) or cultured astroglia (AST) probed with each of the ERK specific probes, as indicated.

D. Independent regulation of individual ERK genes during differentiation of P19 embryocarcinoma cells. Ten micrograms of total RNA from adult rat brain or from undifferentiated P19 cell (STEM), retinoic acid-induced (NEUR) or DMSO-induced (MUSC) were used to prepare replicate Northern blots which were probed as indicated. LANGFR signifies a probe for the low-affinity NGF receptor, the GAPDH control probe verifies that equal amounts of RNA were loaded in each lane.

FIGURE 7. Expression of active ERK2 in _E_. _coli_.

A. Silver stained gels of equal amounts of protein from lysates of E. coli expressing ERK2 or vector alone. The arrow denotes recombinant ERK2.

B. Immunoblot with antiserum 837 of the same amount of E. coli extracts shown in A and about 40 ng of partially purified ERK1.

C. Silver stain (left) and autophosphorylation (right) of 162, 270, or 540 ng of purified recombinant ERK2.

D. Kinase activity of purified recombinant ERK2 incubated for 0, 15, 30, 45, and 60 minutes with MBP.

FIGURE 8. Specificity of antipeptide antibodies.

A. Coomassie blue stain of 100 μg of soluble protein from PC12 cells and adult rat brain.

B. Immunoblot of partially purified ERK1, recombinant ERK2, and 100 μg of soluble protein from PC12 cells, 100 μg of soluble (s) and particulate (p) protein from embryonic brain (EM BR) and adult brain (AD BR) (prepared as described in Boulton et al., 1991, Biochem. 30:278-286) with antiserum 956.

C. Duplicate blot probed with antiserum 837.

FIGURE 9. Immunoprecipitation of ^{32}P-labeled ERK proteins from insulin-stimulated Rat 1 HIRc B cells and NGF-stimulated PC12 cells.

A. ERK1 was immunoprecipitated with antiserum 837 from ^{32}P-labeled (left) Rat 1 HIRc B cells with (+) or without (-) exposure to insulin and (right) PC12 cells with (+) or without (-) exposure to NGF. Tick marks indicate molecular weight standards of 116, 84, 58, 48.5, 36.5, and 26.6 kDa.

B. As in part A with and without NGF but with denaturing immunoprecipitation.

C. Phosphoamino acid analysis of immunoprecipitated ERK1 from NGF-treated PC12 cells. The positions of the phosphoamino acid standards are noted. After 4 hours

of labeling, ERK1 was only phosphorylated on serine in the absence of NGF.

FIGURE 10. Immunoblot of immunoprecipitated ERK proteins. ERKs were immunoprecipitated from 1 mg of supernatant protein from insulin-treated (+) or untreated (-) Rat 1 HIRc B cells under denaturing conditions using antiserum 837. The immunoprecipitated proteins were resolved by SDS-PAGE and probed with either antibodies to phosphotyrosine (P-Y) or with ERK antiserum 691. Lanes labeled ERK contain an aliquot of a phenyl-Sepharose fraction containing both ERKs 1 and 2.

FIGURE 11. Chromatography of supernatants from NGF-treated or untreated PC-12 cells on Mono Q. 10 mg of protein from supernatants of PC12 cells either untreated or treated with NGF were chromatographed on a Mono Q column. Kinase activity with MBP is shown in the upper panel. Numbered fractions were precipitated and immunoblotted with the indicated antibody, either 956, 837 or antiphosphotyrosine (αP-Y).

5. DETAILED DESCRIPTION OF THE INVENTION

For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the invention will be divided into the following subsections:

(i) cloning of the MAP2 kinase protein;
(ii) identification of additional members of the MAP2 protein kinase family;
(iii) expression of recombinant MAP2 protein kinase;
(iv) generation of anti-MAP2 protein kinase antibodies;
(v) bioassays for MAP2 kinase activation; and
(vi) utility of the invention.

5.1. CLONING OF THE MAP2 PROTEIN KINASE

According to the present invention, MAP2 protein kinase may be cloned by identifying cloned nucleic acids which contain sequences homologous to known MAP2 kinase sequence, for example, but not limited to, the sequences set forth in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3), and 3B (SEQ ID NO:5), and/or contained in plasmids pBS-rERK1, pBS-rERK2, or pBS-rERK3, as deposited with the ATCC and assigned accession numbers 40808, 40809, and _____, respectively. Alternatively, it may be desirable to obtain such sequence information from purified MAP2 kinase protein.

Purified MAP2 kinase may be obtained from tissues which contain MAP2 kinase activity, including, but not limited to, T lymphocytes, insulin-treated, terminally differentiated 3T3-L1 adipocytes, post-mitotic adrenal chromaffin cells induced to secrete catecholamines, PC12 cells treated with nerve growth factor, brain tissue, or insulin-treated rat 1 HIRc B cells, as well as lower eukaryotes such as sea star and Xenopus laevis oocytes. Purification of MAP2 kinase from PC12 cells appears to parallel purification of MAP2 kinases from insulin treated rat 1 HIRc B cells (FIGURE 2A).

In a specific embodiment of the invention, and not by way of limitation, MAP2 kinase may be purified to a large extent, as follows (Boulton et al., 1991, Biochem. 30:278-286). Cells containing MAP2 kinase may be used to prepare a cell free extract comprising a crude preparation of MAP2 kinase. For example, either PC12 cells may be cultured in DME medium containing 10% fetal bovine serum and 5% horse serum, and then, prior to NGF treatment, may be incubated in serum-free medium for about one hour. NGF at a concentration of about 4 nM may then be added, and the cells may be incubated for 5 minutes. Alternatively, insulin-treated Rat 1 HIRc B cells may be used. The medium

may then be removed and the cells rinsed and scraped into iced homogenization solution that contains 20 mM p-nitrophenylphosphate, 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 50 mM sodium fluoride, 50 μM sodium orthovanadate and 5 mM benzamidine (MAP2 kinase). Equal numbers of dishes of untreated cells may desirably be harvested as controls. All further steps are preferably performed at 4°C. Cells may be broken with 30-50 strokes of a Dounce homogenizer and homogenates may be centrifuged at 4000 x g for 5 minutes. The supernatants may then be centrifuged at 97,000 x g for 60 minutes. The resulting supernatants may then be assayed, preferably immediately, then frozen in liquid nitrogen.

For purification of MAP2 kinase, soluble fractions (225-300 ml) combined from 150 to 200 150-cm^2 dishes of insulin-treated Rat 1 HIRc Bell cells may be adjusted to a conductivity of 3.5 mS (with water) and to concentrations of 40 μM cAMP, 0.5 mM phenylmethylsulfonylfluoride and 0.1 μM pepstatin prior to chromatography on a Q-Sepharose column (1.5 x 19 cm). The column may be washed with 4 to 5 volumes of buffer A (10% glycerol, 25 mM Tris-HCl, pH 7.5, 50 μM sodium orthovanadate, 1 mM dithiothreitol, 50 mM NaF, 20 mM β-glycerol phosphate, 1 mM EGTA, 10 mM benzamidine, 10 mM p-nitrophenylphosphate, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1 μM pepstatin) containing 40 μM cAMP. Protein may then be eluted by a gradient of 0-0.4 M NaCl in buffer A. Fractions containing stimulated MAP2 kinase activity may be pooled and applied to a phenyl-Sepharose column (1.5 x 18 cm). The column may then be washed with 5 column volumes of buffer A containing 0.25 M NaCl and protein may be eluted with a descending gradient of 0.25-0.025 M NaCl plus an ascending gradient of 0-65% ethylene glycol in buffer A without glycerol. Kinase activity may be pooled from the phenyl-Sepharose column and applied directly to a 5 ml column (1.5 x 3 cm) of S-Sepharose

followed by a 5 ml column of phosphocellulose (1.5 x 3 cm). In both cases, unadsorbed material containing MAP2 kinase activity and 2 column volumes of wash may be collected. The MAP2 kinase activity from the phosphocellulose column may be applied directly to a QAE-Sepharose column (1 x 24 cm). The column may be washed with 5 volumes of buffer A and protein may be eluted with a gradient of 0-0.4 M NaCl in buffer A. The fractions containing MAP2 kinase activity may then be pooled, Brij-58 may be added to give a final concentration of 0.01% (included in all subsequent steps), and the sample may be concentrated by ultrafiltration to 1.5-2 ml in order to load onto an Ultrogel AcA54 column (1 x 112 cm) equilibrated in buffer A containing 0.2 M NaCl and 0.01% Brij-58. Fractions from the gel filtration column may be collected into tubes containing 2.4 mM leupeptin. The fractions containing activity may be concentrated and diluted with 25 mM Tris, pH 7.5, 1 mM DTT, 10 mM sodium phosphate, 0.1 μM pepstatin, 0.5 mM phenylmethyl sulfonyl fluoride containing 0.01% Brij-58 until the conductivity is reduced to 3 mS and then may be applied to DEAE-cellulose (0.7 x 18 cm). The activity may be eluted with a gradient of 0-0.25 M NaCl in buffer A. Fractions containing activity may be pooled, and, as necessary, concentrated and diluted as above to apply to either a Mono Q HR 5/5 or a Q-Sepharose (0.5 x 9 cm) column. The MAP2 kinase activity may be eluted with a gradient of 0-0.25 M NaCl (from Mono Q) in buffer A. Fractions may be assayed and then immediately frozen in liquid nitrogen.

Purified MAP2 kinase may then be digested with trypsin and the resulting peptides subjected to HPLC (Abersold et al., 1987, Proc. Natl. Acad. Sci. U.S.A. <u>84</u>:6970-6974) as described in section 6.1, <u>infra</u>. The peptides from one of the resulting peaks may then be subjected to a second chromatographic separation. In order to determine

fragments of MAP2 kinase protein sequence accurately, it may be necessary to perform repeated purification of peptides and to discriminate between major and minor component peptides, as would be recognized by one skilled in the art.

Peptides may be sequenced by any method known in the art. For example, fractions containing the enzyme may be pooled and final concentrations of 0.05% Lubrol and 8.5% trichloroacetic acid (w/v) may be added to precipitate the protein. After washing with acetone,the protein may be dissolved in electrophoresis buffer and 250 pmol may be loaded onto a 10% polyacrylamide gel in SDS. Protein may be electrophoretically transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). The 43 kDa band may be excised for in situ digestion with trypsin (Abersold et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:6970-6974), leaving the minor component, which migrates only slightly faster, on the nitrocellulose. Peptides released from the excised piece of nitrocellulose may be subjected to HPLC on a Model 130A chromatography system (Applied Biosystems, Inc., Foster City, CA) equipped with a 2.1 x 100 mm Applied Biosystems RP-300 column. Separations may be performed in 0.1% trifluoroacetic acid at a flow rate of 50 μl/min using a gradient of 0-70% (v/v) acetonitrile of 100-min duration. Absorbency of the eluate may be monitored at 214 nm and the components that eluted may be collected manually. Peptides may be dried onto 1 cm discs of Whatman GF/C paper and sequenced using an Applied Biosystems, Inc. Model 470A amino acid sequencer equipped with a Model 120A phenylthiohydantoin analyzer, according to manufacturer's specifications.

The purification of suitable amounts of MAP2 kinase protein to permit microsequencing makes possible the cloning of a MAP2 kinase cDNA. A strategy for such cloning might be to generate a complementary oligonucleotide probe,

based on a segment of known amino acid sequence, and to use this probe to screen cDNA libraries generated from tissue presumed to synthesize mRNA encoding MAP2 kinase as follows. First, the amino acid sequence derived from purified MAP2 kinase protein may be used to deduce oligonucleotide primers which may be generated and used in standard screening techniques or used in polymerase chain reaction (PCR) (Saiki et al., 1985, Science 230:1350-1354). Because of the degeneracy of the genetic code, in which several triplets may specify the same amino acid, several oligonucleotides should be synthesized for a given amino acid sequence, in order to provide for multiple potential nucleotide sequence combinations; the resulting oligonucleotides are referred to as degenerate primers. For example, in a specific embodiment of the invention, a series of degenerate oligonucleotides may be synthesized that correspond to the coding or anti-coding strands for segments of tryptic peptide sequences obtained from purified MAP2 kinase protein. The oligonucleotides may desirably contain non-degenerate tails at their 5′ ends; the tail of each coding strand oligonucleotide may contain, for example, an EcoR1 restriction site, while the tail of each anti-coding strand oligonucleotide may, for example, contain a Sall restriction site. Each coding strand oligonucleotide may then be combined with each anti-coding oligonucleotide in individual PCR reactions using cDNA from Rat 1 cells as template; the PCR reactions and the preparation of the genomic and cDNA templates may then be performed as described in Maisonpierre, C. et al., 1990, Science 247:1446-1451 and Bothwell, A., Yancopoulos, G. and Alt, F., 1990, "Methods for Cloning and Analysis of Eukaryotic Genes", Jones and Bartlett, Boston, MA. The amplified product obtained using, for example, the QYIGEG coding oligonucleotide and the DLKPSN anti-coding oligonucleotide (designated QYDL) may then be isolated

using a Sephadex G-50 spin column, digested with EcoR1 and Sal1, gel purified using 2% Nusieve (FMC Bioproducts), and subcloned into a vector comprising suitable restriction sites, such as the pGEM4Z vector (Promega).

A suitable library, believed to be likely to contain a MAP2 kinase gene, may then be screened with labeled nucleic acid probe (for example, subcloned PCR product radiolabeled using a PCR-based protocol (Maisonpierre et al., 1990, Science 247:1446-1451)). Examples of a suitable library would include a rat brain or T lymphocyte cDNA library or a cDNA library produced from PC12 cells or post-mitotic adrenal chromaffin cells, to name but a few. Hybridization conditions may be performed as described in Maisonpierre et al. (1990, Science 247:1446-1451) or using any standard techniques; washing of filters may preferably be performed first at low stringency (2 X SSC (20 mM sodium citrate, pH 7.0, 0.15 M NaCl), 0.1% SDS at 60°C) and then at high stringency (0.2 X SSC, 0.1% SDS at 60°C).

Once obtained, a MAP2 kinase gene may be cloned or subcloned using any method known in the art. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322, pUC, or Bluescript® (Stratagene) plasmid derivatives. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc.

The MAP2 kinase gene may be inserted into a cloning vector which can be used to transform, transfect, or infect appropriate host cells so that many copies of the gene sequences are generated. This can be accomplished by ligating the DNA fragment into a cloning vector which has

complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. It may prove advantageous to incorporate restriction endonuclease cleavage sites into the oligonucleotide primers used in polymerase chain reaction to facilitate insertion into vectors. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and MAP2 kinase gene may be modified by homopolymeric tailing.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated MAP2 kinase gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

According to a preferred embodiment of the invention, once a cDNA-derived clone encoding MAP2 kinase has been generated, a genomic clone encoding MAP2 kinase may be isolated using standard techniques known in the art. For example, a labeled nucleic acid probe may be derived from the MAP2 kinase clone, and used to screen genomic DNA libraries by nucleic acid hybridization, using, for example, the method set forth in Benton and Davis (1977, Science <u>196</u>:180) for bacteriophage libraries and Grunstein and Hogness (1975, Proc. Natl. Acad. Sci. U.S.A. <u>72</u>:3961-3965) for plasmid libraries. Retrieved clones may then be analyzed by restriction-fragment mapping and sequencing techniques according to methods well known in the art.

Furthermore, additional cDNA clones may be identified from a cDNA library using the sequences obtained according to the invention.

5.2. IDENTIFICATION OF ADDITIONAL MEMBERS OF THE MAP2 PROTEIN KINASE FAMILY

The present invention provides for recombinant nucleic acid molecules corresponding to mammalian nucleic acids which are homologous to the nucleic acid sequences substantially as depicted in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5) or portions thereof of at least 10 nucleotides.

According to the present invention, by screening a DNA library (comprising genomic DNA or, preferably, cDNA) with oligonucleotides corresponding to MAP2 kinase sequence derived either from protein sequence data or from the nucleic acid sequence set forth in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5), clones may be identified which encode distinct members of the MAP2 kinase family, as exemplified in Section 7, infra, in which additional members of the MAP2 kinase family were identified. By decreasing the stringency of hybridization, the chances of identifying somewhat divergent members of the family may be increased. It may also be desirable to use sequences substantially shared by members of the MAP2 kinase family which have been sequenced preferably, for example, sequences from domains V or VI; such highly conserved regions may be particularly useful in identifying additional members of the MAP2 kinase family. Library screening may be performed using, for example, the hybridization technique of Benton and Davis (1977, Science 196:180) or Grunstein and Hogness (1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961-3965). Clones identified by hybridization may then be further analyzed, and new family members may be identified by restriction fragment mapping

and sequencing techniques according to methods well known in the art.

It may be desirable to utilize polymerase chain reaction (PCR) technology (Saiki et al., 1985), Science 230:1350-1354) to identify additional members of the MAP2 protein kinase family. For example, sense and antisense primers corresponding to known MAP2 protein kinase sequence (which preferably appears to be conserved among characterized members of the MAP2 protein kinase family) may be used in PCR, with cDNA obtained from cells which produce MAP2 kinase as template. It may be desirable to design these primers such that they include restriction enzyme cleavage sites which may facilitate the insertion of the products of PCR into appropriate cloning vectors. The products of PCR may be inserted into suitable vectors as set forth in Section 5.1, supra, and the resulting clones may then be screened for new family members. Such screening may be performed using standard techniques, including hybridization analysis using probes corresponding to known MAP2 kinase sequence. For example, a series of probes representing different regions of an already characterized MAP2 kinase protein may be hybridized at low stringency to duplicate filters carrying DNA from clones generated using PCR, as outlined above. It may be observed that various clones may hybridize to some probes, but not others. New family members may also be identified by increasing the stringency of the hybridization conditions, wherein new members not identical to probes derived from known members (e.g. ERK1, ERK2 or ERK3) would hybridize less strongly at higher stringency. Alternatively, new family members may be identified by restriction mapping or sequencing analysis using standard techniques to reveal differences in restriction maps or sequences relative to known family members.

5.3. EXPRESSION OF RECOMBINANT MAP2 PROTEIN KINASE

The present invention provides for recombinant MAP2 protein kinase molecule comprising the amino acid sequence substantially as depicted in FIGURES 2B (SEQ ID NO:2), 3A (SEQ ID NO:4) or 3B (SEQ ID NO:6), or a portion thereof, which has a molecular weight, by SDS-PAGE, of between about 41 and 48 kDa, or about 62-63 kDA, or which comprises a portion homologous to the yeast FUS3 or KSS1 protein kinase as well as a short amino terminal extension or which has a carboxy terminal extension of about 180 amino acids. The present invention also provides for mammalian MAP2 protein kinases homologous to the above-mentioned molecules.

In order to express recombinant MAP2 kinase, the nucleotide sequence coding for a MAP2 kinase protein, or a portion thereof, can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translation signals can also be supplied by the native MAP2 kinase gene and/or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression elements of these vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene

consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleic acid sequence encoding MAP2 kinase protein or peptide fragment may be regulated by a second nucleic acid sequence so that MAP2 kinase protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of MAP2 kinase may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control MAP2 kinase expression include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), [the CMV promoter] the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionine gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25), see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phophatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald,

1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Expression vectors containing MAP2 kinase gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted MAP2 kinase gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence

of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the MAP2 kinase gene is inserted within the marker gene sequence of the vector, recombinants containing the MAP2 kinase insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the MAP2 kinase gene product in bioassay systems as described supra, in Section 5.2. However, if cells containing MAP2 kinase expression constructs contain intrinsic MAP2 kinase, activity resulting from the construct can be distinguished from endogenous kinase activity (e.g. put a distinguishing tag on the recombinant molecule) or by subtracting background levels of endogenous kinase.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be

elevated in the presence of certain inducers; thus, expression of the genetically engineered MAP2 kinase protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of the heterologous MAP2 kinase protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extents.

Once a recombinant which expresses the MAP2 kinase gene is identified, the gene product should be analyzed. This can be achieved by assays based on the physical or functional properties of the product.

Once the MAP2 kinase protein is identified, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

The presence of functional MAP2 kinase activity may be determined as set forth in section 5.5, infra.

5.3.1. MAP2 GENE KINASE GENES AND PROTEINS

Using the methods detailed supra and in Example Sections 6 and 7, infra, the following nucleic acid sequences were determined, and their corresponding amino acid sequences deduced. The sequences of two rat MAP2 kinase cDNAs were determined, and are depicted in FIGURES

2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5). Each of these sequences, or their functional equivalents, can be used in accordance with the invention. Additionally, the invention relates to MAP2 kinase genes and proteins isolated from porcine, ovine, bovine, feline, avian, equine, or canine, as well as primate sources and any other species in which MAP2 kinase activity exists. The present invention also provides for ERK4, as identified and described in Section 7, <u>infra</u>, which corresponds to a protein having a molecular weight of about 45 kDa. The invention is further directed to homologous subsequences of MAP2 kinase nucleic acids comprising at least ten nucleotides, such subsequences comprising hybridizable portions of the MAP2 kinase sequence which have use, e.g., in nucleic acid hybridization assays, Southern and Northern blot analyses, etc. The invention also provides for MAP2 kinase proteins, fragments and derivatives thereof, according to the amino acid sequences set forth in FIGURES 2B (SEQ ID NO:2), 3A (SEQ ID NO:4) and 3B (SEQ ID NO:6) or their functional equivalents and for proteins homologous to such proteins, such homology being of at least about 30 percent. The invention also provides fragments or derivatives of MAP2 kinase proteins which comprise antigenic determinant(s) or which are functionally active or which are at least six amino acids in length. As used herein, functionally active shall mean having the capacity to phosphorylate MAP2 or other relevant substrates (e.g. MBP, S6 kinase; see Section 5.5, <u>infra</u>).

For example, the nucleic acid sequences depicted in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5) can be altered by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as depicted in FIGURES 2B (SEQ

ID NO:2), 3A (SEQ ID NO:4) and 3B (SEQ ID NO:6) may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of the MAP2 kinase genes depicted in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5) which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the MAP2 kinase proteins, or fragments or derivatives thereof, of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence substantially as depicted in FIGURES 2B (SEQ ID NO:2), 3A (SEQ ID NO:4) and 3B (SEQ ID NO:6) including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are MAP2 kinase proteins or fragments or derivatives thereof which are differentially modified during or after translation, e.g., by phosphorylation, glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. For example, it

may be desirable to modify the sequence of a MAP2 kinase such that specific phosphorylation, i.e. serine threonine, is no longer required or as important.

In addition, the recombinant MAP2 kinase encoding nucleic acid sequences of the invention may be engineered so as to modify processing or expression of MAP2 kinase. For example, and not by way of limitation, a signal sequence may be inserted upstream of MAP2 kinase encoding sequences to permit secretion of MAP2 kinase and thereby facilitate harvesting or bioavailability.

Additionally, a given MAP2 kinase can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, et al., 1978, J. Biol. Chem. 253:6551), use of TAB® linkers (Pharmacia), etc.

5.4. GENERATION OF ANTI-MAP2 PROTEIN KINASE ANTIBODIES

According to the invention, MAP2 kinase protein, or fragments or derivatives thereof, may be used as immunogen to generate anti-MAP2 kinase antibodies. By providing for the production of relatively abundant amounts of MAP2 kinase protein using recombinant techniques for protein synthesis (based upon the MAP2 kinase nucleic acid sequences of the invention), the problem of limited quantities of MAP2 kinase has been obviated.

To further improve the likelihood of producing an anti-MAP2 kinase immune response, the amino acid sequence of MAP2 kinase may be analyzed in order to identify portions of the molecule which may be associated with

increased immunogenicity. For example, the amino acid sequence may be subjected to computer analysis to identify surface epitopes which present computer-generated plots of hydrophilicity, surface probability, flexibility, antigenic index, amphiphilic helix, amphiphelic sheet, and secondary structure of MAP2 kinase. Alternatively, the deduced amino acid sequences of MAP2 kinase from different species could be compared, and relatively non-homologous regions identified; these non-homologous regions would be more likely to be immunogenic across various species.

For preparation of monoclonal antibodies directed toward MAP2 kinase, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452).

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of MAP2

kinase. For the production of antibody, various host animals can be immunized by injection with MAP2 kinase protein, or fragment or derivative thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and, Corynebacterium parvum.

A molecular clone of an antibody to a MAP2 kinase epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

The present invention provides for antibody molecules as well as fragments of such antibody molecules.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

5.5. BIOASSAYS FOR MAP2 KINASE ACTIVITY

The activity of MAP2 kinase may be measured using any suitable kinase assay known in the art. For example, and not by way of limitation, the method described in (Boulton et al., 1990, J. Biol. Chem. 265:2713-2719) as follows. The assay for phosphorylation of MAP2 may contain 30 mM Hepes, pH 8, 50 μM ATP (1-50 cpm/fmol), 1 mM dithiothreitol, 1 mM benzamidine, 10 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 3 μg MAP2 and no more than about 10 μg sample protein in a final volume of 30μl for 10 minutes at 30°C. The amount of MAP2 in the assay (100 μg/ml) may be chosen for convenience of analysis both by SDS-PAGE and by precipitation. The enzyme is not saturated with substrate even at 1.36 mg MAP2/ml. However, with 100 μg MAP2/ml enzyme activity may be expected to be linear with time for at least 30 minutes. All samples except for unfractionated supernatants may be routinely assayed as above in the presence of 1 mg of bovine serum albumin. Assays may be terminated by the addition of 10% trichloroacetic acid and precipitates may be collected on glass fiber filters. All assays except as noted above may be terminated by the addition of 0.25 volume of 0.3 M Tris-HCl, pH 6.9, containing 2 M mercaptoethanol, 50% glycerol and 10% SDS and analyzed by electrophoresis in SDS using 5% (MAP2) polyacrylamide gels. The gels may be stained with Coomassie blue, destained in 10% methanol and 10% acetic acid, dried and subjected to autoradiography at -80°C using Kodak XS-5 or BB-5 film with Dupont Quanta III intensifying screens. Substrate bands may be excised from gels and ^{32}P may be quantitated using liquid scintillation counting.

5.6. UTILITY OF THE INVENTION

The present invention may be utilized to provide unique model systems for the study of mechanisms of

hormones and other cellular factors, and may also be used in methods for screening compounds for hormone/cellular factor activity and to identify agents which function as agonists or antagonists.

According to various embodiments of the invention, recombinant MAP2 kinase molecules can be used to create novel model systems for the study of mechanisms of hormones and other cellular factors. For example, and not by way of limitation, the recombinant molecules of the invention can be incorporated into cells or organisms such that higher than normal amounts of MAP2 kinase are produced, so that the effects of hyperactivation of MAP2 kinase may be evaluated. Overproduction of MAP2 kinase may identify aspects of the hormonal/cellular factor response related to MAP2 kinase activity, particularly when evaluated in comparison to cells or organisms which produce normal amounts of MAP2 kinase.

Alternatively, recombinant MAP2 kinase molecules may be engineered such that cells or organisms comprising the recombination molecules produce a mutant form of MAP2 kinase which may, for example, lack the serine/threonine kinase activity of normal MAP2 kinase. The mutant kinase may, on a concentration basis, overshadow, or titrate out, the effects of normal MAP2 kinase and thereby create cells or organisms with a functional aberrancy of MAP2 kinase function. It is also envisioned that such mutant nucleic acid sequences may result in mutation of the endogenous MAP2 kinase gene, for example, by homologous recombination, creating true MAP2 kinase mutants. In light of the high levels of expression of MAP2 kinase encoding mRNA in the central nervous system, and the role of MAP2 in forming neurofibrillary tangles, it may be possible to generate a transgenic non-human animal which expresses a mutant MAP2 kinase molecule in its central nervous system (e.g. via a brain-specific promoter sequence) and which may serve as an

5.5. BIOASSAYS FOR MAP2 KINASE ACTIVITY

The activity of MAP2 kinase may be measured using any suitable kinase assay known in the art. For example, and not by way of limitation, the method described in (Boulton et al., 1990, J. Biol. Chem. 265:2713-2719) as follows. The assay for phosphorylation of MAP2 may contain 30 mM Hepes, pH 8, 50 μM ATP (1-50 cpm/fmol), 1 mM dithiothreitol, 1 mM benzamidine, 10 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 3 μg MAP2 and no more than about 10 μg sample protein in a final volume of 30μl for 10 minutes at 30°C. The amount of MAP2 in the assay (100 μg/ml) may be chosen for convenience of analysis both by SDS-PAGE and by precipitation. The enzyme is not saturated with substrate even at 1.36 mg MAP2/ml. However, with 100 μg MAP2/ml enzyme activity may be expected to be linear with time for at least 30 minutes. All samples except for unfractionated supernatants may be routinely assayed as above in the presence of 1 mg of bovine serum albumin. Assays may be terminated by the addition of 10% trichloroacetic acid and precipitates may be collected on glass fiber filters. All assays except as noted above may be terminated by the addition of 0.25 volume of 0.3 M Tris-HCl, pH 6.9, containing 2 M mercaptoethanol, 50% glycerol and 10% SDS and analyzed by electrophoresis in SDS using 5% (MAP2) polyacrylamide gels. The gels may be stained with Coomassie blue, destained in 10% methanol and 10% acetic acid, dried and subjected to autoradiography at -80°C using Kodak XS-5 or BB-5 film with Dupont Quanta III intensifying screens. Substrate bands may be excised from gels and ^{32}P may be quantitated using liquid scintillation counting.

5.6. UTILITY OF THE INVENTION

The present invention may be utilized to provide unique model systems for the study of mechanisms of

27. A six amino acid portion of the recombinant MAP2 protein kinase of claim 21.

28. The recombinant nucleic acid molecule of claim 1 comprising a nucleic acid sequence substantially as depicted in Figure 3B (SEQ ID NO:5).

29. The recombinant nucleic acid molecule of claim 28 as comprised in the vector pBS-rERK3, deposited with the ATCC and having accession number 75009.

30. An isolated recombinant nucleic acid molecule comprising a mammalian nucleic acid which is capable of hybridizing to a nucleic acid molecule having a nucleic acid sequence substantially as depicted in Figure 3B (SEQ ID NO:5), or a portion thereof.

31. An isolated recombinant nucleic acid molecule which encodes a protein having the amino acid sequence substantially as depicted in Figure 3B (SEQ ID NO:6), or a portion consisting of at least six amino acids thereof.

32. An organism containing the recombinant nucleic acid molecule of claim 28.

33. A cell containing the recombinant nucleic acid molecule of claim 28 or 31.

34. The recombinant nucleic acid molecule of claim 31 which further comprises a nucleic acid sequence capable of controlling gene expression.

35. A substantially purified MAP2 protein kinase molecule comprising the amino acid sequence substantially as depicted in Figure 3B (SEQ ID NO:6), or a portion consisting of at least six amino acids thereof.

36. A substantially purified mammalian protein kinase homologous to the recombinant MAP2 protein kinase of claim 35.

37. A substantially purified MAP2 protein kinase encoded by the recombinant nucleic acid molecule of claim 29 or 34.

38. A method of detecting the presence of a compound having nerve growth factor-like activity, comprising:

(i) culturing cells that produce a MAP2 protein kinase that is activated by nerve growth factor in the presence of a compound suspected of having nerve growth factor-like activity; and

(ii) detecting changes in the level of the MAP2 protein kinase activity,

wherein an increase in activity is indicative of the presence of nerve growth factor-like activity.

39. The method of claim 38 in which the cells are PC12 cells.

40. The method of claim 38 in which the cells contain a recombinant nucleic acid molecule encoding a mammalian MAP2 kinase.

41. A method of detecting the presence of a compound having insulin-like activity, comprising:

(i) culturing cells that produce a MAP2 protein kinase that is activated by insulin in the presence of a compound suspected of having insulin-like activity; and

(ii) detecting changes in the level of the MAP2 protein kinase activity,

wherein an increase in activity is indicative of the presence of insulin-like activity.

42. The method of claim 41 in which the cells are Rat 1 HIRc B cells.

43. The method of claim 41 in which the cells contain a recombinant nucleic acid molecule encoding a mammalian MAP2 kinase.

44. A method of detecting the presence of a compound which directly or indirectly causes a change in the levels of a MAP2 protein kinase activity, comprising:

(i) culturing cells that produce a MAP2 protein kinase in the presence of a compound; and

(ii) detecting changes in the level of the MAP2 protein kinase activity,

wherein a change in activity is indicative of the presence of the compound.

45. The method of claim 43 in which the compound is a neurotrophin molecule, and the change in activity is an increase in activity.

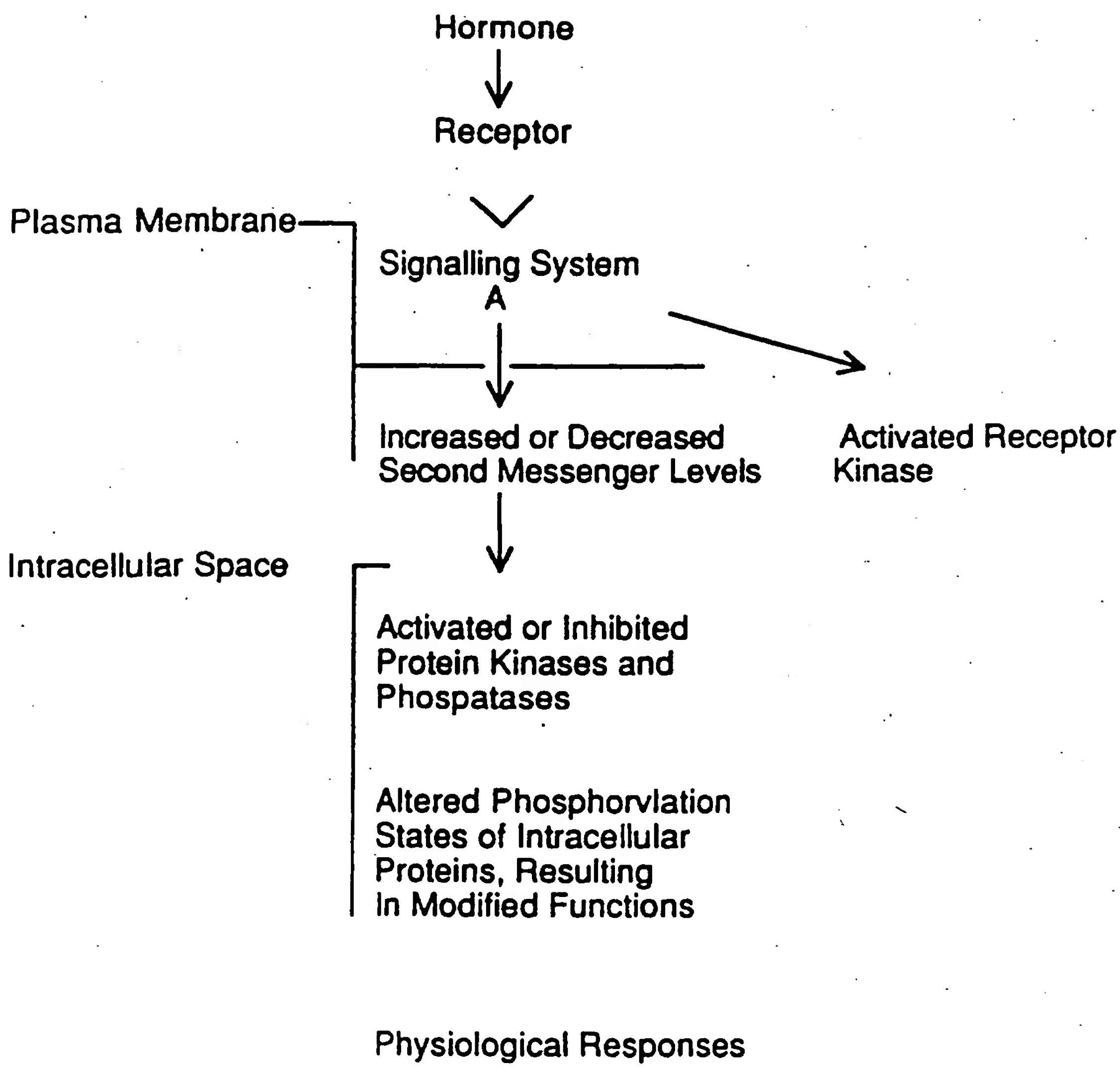
Hormone
Receptor
Plasma Membrane
Signalling System
A
Increased or Decreased
Second Messenger Levels
Activated Receptor
Kinase
Intracellular Space
Activated or Inhibited
Protein Kinases and
Phospatases
Altered Phosphorvlation
States of Intracellular
Proteins, Resulting
In Modified Functions
Physiological Responses

FIG. 1

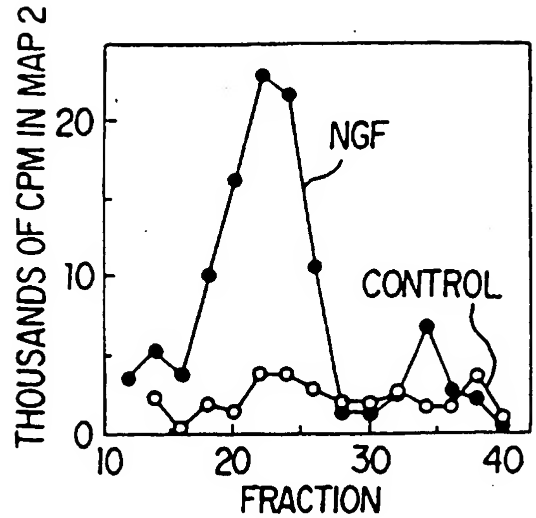

FIG. 2A(A1)

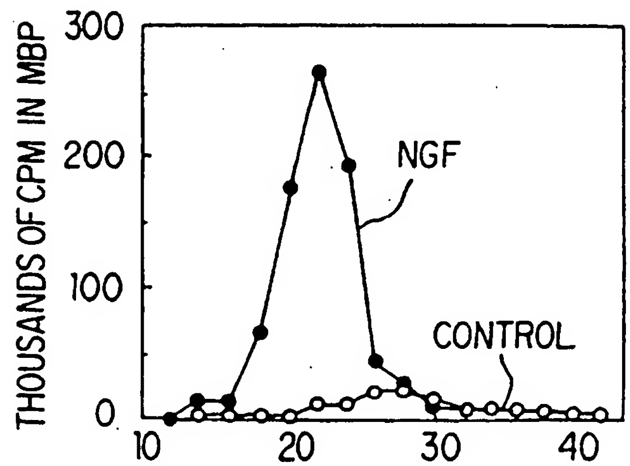

FIG. 2A(A2)

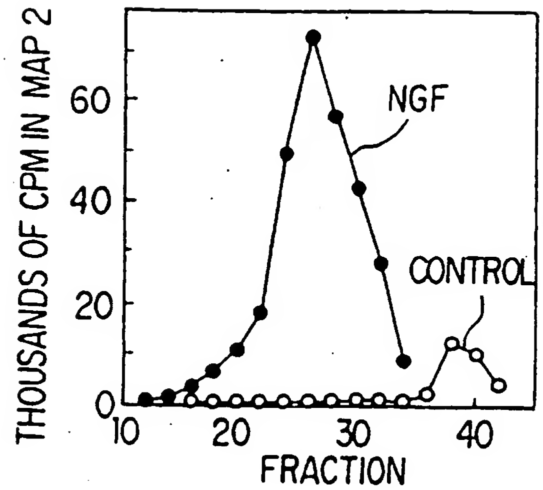

FIG. 2A(B)

ACA-54 OF
PHENYL SEPHAROSE

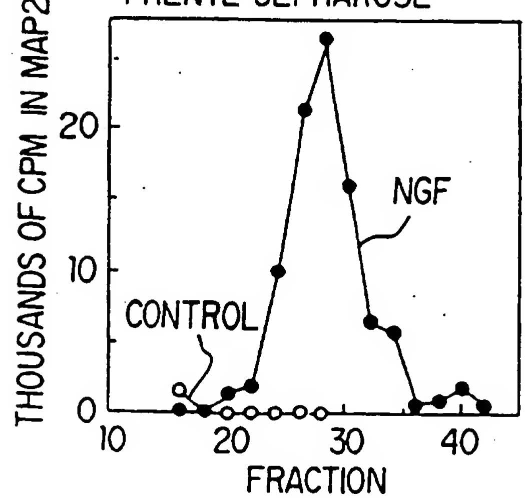

FIG. 2A(C)

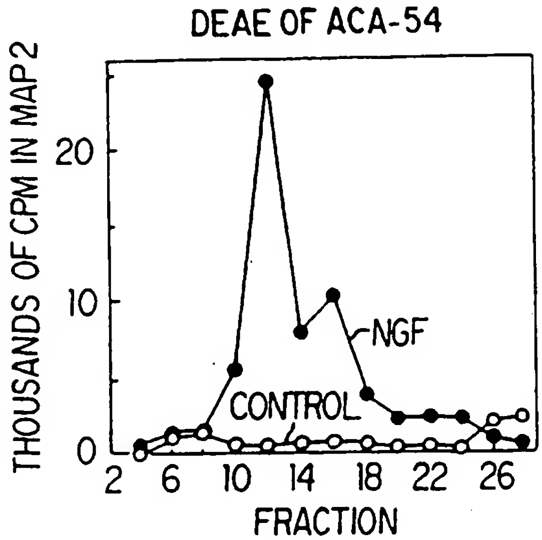

FIG. 2A(D)

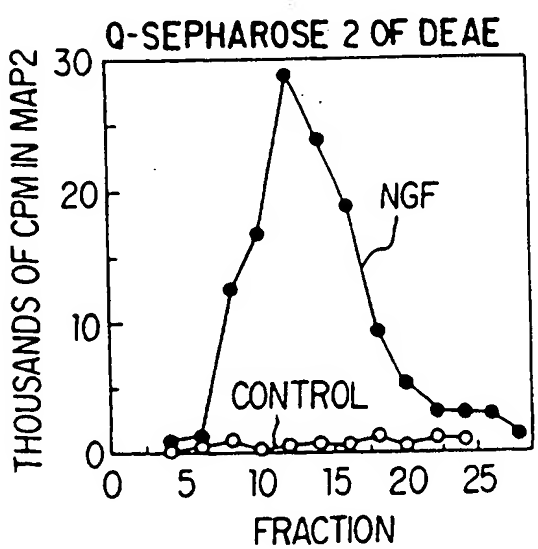

FIG. 2A(E)

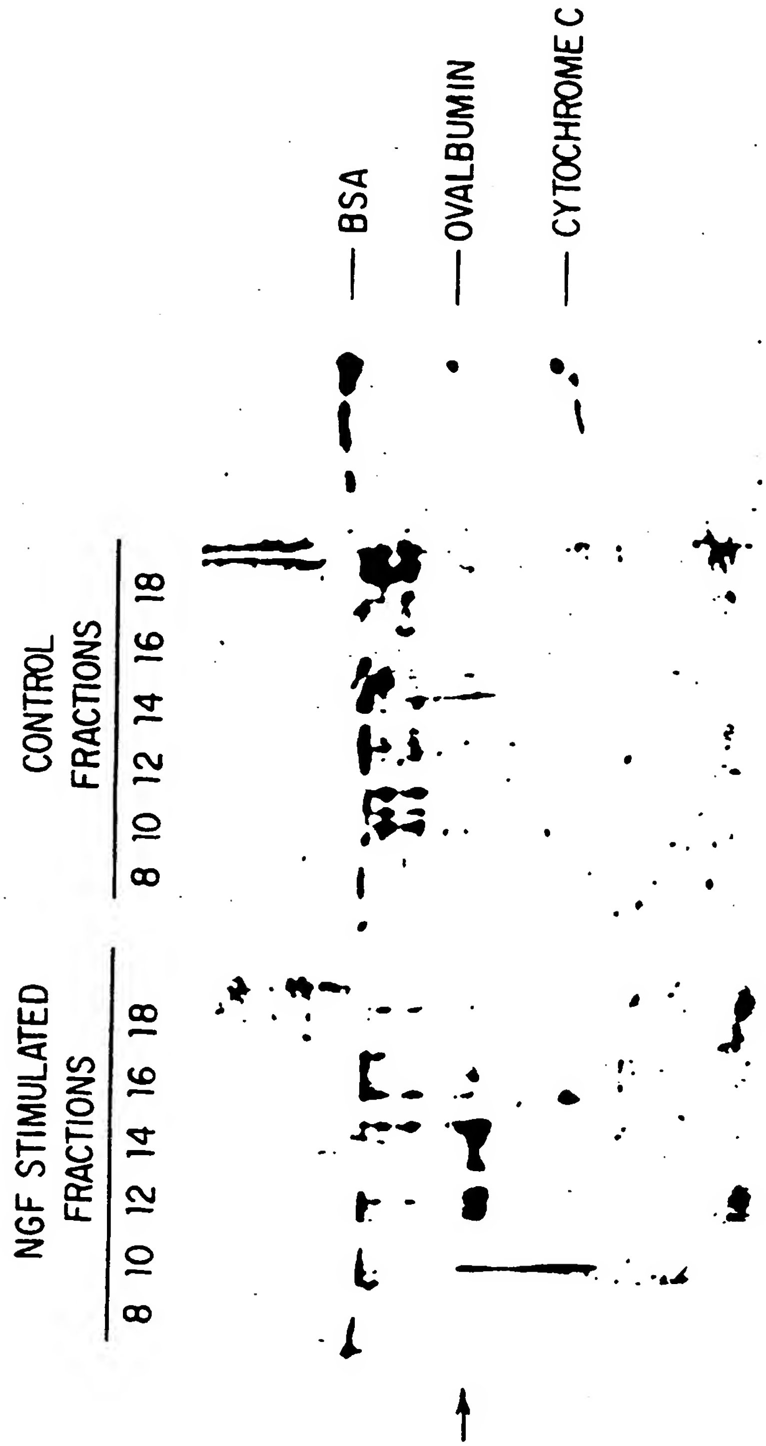
NGF STIMULATED
FRACTIONS
8 10 12 14 16 18
CONTROL
FRACTIONS
8 10 12 14 16 18
BSA
OVALBUMIN
CYTOCHROME C

FIG. 2A(F)

Arg Gly Thr
AGG GGA ACT

Ala Gly Val Val Pro Val Val Pro Gly Glu Val Glu Val Val
GCT GGG GTC GTC CCG GTG GTC CCC GGG GAG GTG GAG GTG GTG

Lys Gly Gln Pro Phe Asp Val Gly Pro Arg Tyr Thr Gln
AAG GGG CAG CCA TTC GAC GTG GGC CCA CGC TAC ACG CAG

Leu Gln Tyr Ile Gly Glu Gly Ala Tyr Gly Met Val Ser Ser
CTG CAG TAC ATC GGC GAG GGC GCG TAC GGC ATG GTC AGC TCA

Ala Tyr Asp His Val Arg Lys Thr Arg Val Ala Ile Lys
GCA TAT GAC CAC GTG CGC AAG ACC AGA GTG GCT ATC AAG

Lys Ile Ser Pro Phe Glu His Gln Thr Tyr Cys Gln Arg Thr
AAG ATC AGC CCC TTC GAG CAT CAA ACC TAC TGT CAG CGC ACG

Leu Arg Glu Ile Gln Ile Leu Leu Gly Phe Arg His Glu
CTG AGA GAA ATC CAG ATC TTG CTC GGA TTC CGC CAT GAG

Asn Val Ile Gly Ile Arg Asp Ile Leu Arg Ala Pro Thr Leu
AAT GTC ATA GGC ATC CGA GAC ATC CTC AGA GCA CCC ACC CTG

Glu Ala Met Arg Asp Val Tyr Ile Val Gln Asp Leu Met
GAA GCC ATG AGA GAT GTT TAC ATT GTT CAG GAC CTC ATG

Glu Thr Asp Leu Tyr Lys Leu Leu Lys Ser Gln Gln Leu Ser
GAG ACG GAC CTG TAC AAG CTG CTA AAG AGC CAG CAG CTG AGC

Asn Asp His Ile Cys Tyr Phe Leu Tyr Gln Ile Leu Arg
AAT GAC CAC ATC TGC TAC TTC CTC TAC CAG ATC CTC CGG

Gly Leu Lys Tyr Ile His Ser Ala Asn Val Leu His Arg Asp
GGC CTC AAG TAC ATA CAC TCG GCC AAT GTG CTG CAC CGG GAC

Leu Lys Pro Ser Asn Leu Leu Ile Asn Thr Thr Cys Asp
CTG AAG CCC TCC AAT CTG CTT ATC AAC ACC ACC TGC GAC

FIG.2B(i)

* * *
Leu Lys Ile Cys Asp Phe Gly Leu Ala Arg Ile Ala Asp Pro
CTT AAG ATC TGT GAT TTT GGC CTT GCC CGG ATT GCT GAC CCT

Glu His Asp His Thr Gly Phe Leu Thr Glu Tyr Val Ala
GAG CAC GAC CAC ACT GGC TTT CTG ACC GAG TAT GTG GCC

* * *
Thr Arg Trp Tyr Arg Ala Pro Glu Ile Met Leu Asn Ser Lys
ACA CGC TGG TAC CGA GCC CCA GAG ATC ATG CTT AAC TCC AAG

* *
Gly Tyr Thr Lys Ser Ile Asp Ile Trp Ser Val Gly Cys
GGC TAC ACC AAA TCC ATT GAC ATC TGG TCT GTG GGC TGC

Ile Leu Ala Glu Met Leu Ser Asn Arg Pro Ile Phe Pro Gly
ATT CTG GCT GAG ATG CTC TCC AAC CGG CCT ATC TTC CCC GGC

Lys His Tyr Leu Asp Gln Leu Asn His Ile Leu Gly Ile
AAG CAC TAC CTG GAC CAG CTC AAC CAC ATT CTA GGT ATA

Leu Gly Ser Pro Ser Gln Glu Asp Leu Asn Cys Ile Ile Asn
CTG GGT TCC CCA TCC CAA GAG GAC CTA AAT TGT ATC ATT AAC

Met Lys Ala Arg Asn Tyr Leu Gln Ser Leu Pro Ser Lys
ATG AAG GCC CGA AAC TAC CTA CAG TCT CTG CCC TCT AAA

Thr Lys Val Ala Trp Ala Lys Leu Phe Pro Lys Ser Asp Ser
ACC AAG GTG GCT TGG GCC AAG CTT TTT CCC AAA TCT GAC TCC

Lys Ala Leu Asp Leu Leu Asp Arg Met Leu Thr Phe Asn
AAA GCT CTT GAC CTG CTG GAC CGG ATG TTA ACC TTT AAC

*
Pro Asn Lys Arg Ile Thr Val Glu Glu Ala Leu Ala His Pro
CCA AAC AAG CGC ATC ACA GTA GAG GAA GCA CTG GCT CAC CCT

Tyr Leu Glu Gln Tyr Tyr Asp Pro Thr Asp Glu Pro Val
TAC CTG GAA CAG TAC TAT GAT CCG ACA GAT GAA CCA GTG

Ala Glu Glu Pro Phe Thr Phe Asp Met Glu Leu Asp Asp Leu
GCT GAG GAG CCA TTC ACC TTT GAC ATG GAG CTG GAT GAT CTC

FIG.2B(ii)

```
Pro Lys Glu Arg Leu Lys Glu Leu Ile Phe Gln Glu Thr
CCC AAG GAG CGG CTG AAG GAG CTG ATC TTC CAA GAG ACA
Ala Arg Phe Gln Pro Gly Ala Pro Glu Ala Pro END CAAGAAC
GCC CGC TTC CAG CCA GGG GCA CCA GAG GCC CCC TAA

AGACACCCCTGTCCTTTTGGACCTGGTCTGCTCTACCTGCTCCTTCTCTGCA

GATTGTTAGAAAATGAACTTTGCTCAACCCGGACCCCGGCAGCCCAGGCTGGACC

AAGGGTGGGCCTGGCACCCCTCTCACTCTGCTGGGGTCTCCTCGTTCAAGAG

GCTTCTCCCACTCCAGTCCCCTGCCCCATCTCCCCTTGACCTGAGTGATGAGGTG

GTCCCAGAGCTGATCTCTGCTGCTGTGTCTTTATCTATCCCTGCTAGCCCCA

GCTCTGGTAGACGGTTCTGGAATGGAAGGGCTATGACCGCCCTAGGACCTGTGCT

ACAGAGGGGTGGAGGGCACTGAGTAGGCTAAGCTCTGCCCTACTCATCCTGT

TGGAACCCCACCCCATTTTCCCTGACAGAACATTCCTAAATCTCAAGGGCTAGTT

TCCCTGAGGAGCCAGCCTAGGCCTAACCCTCTCCCTCTCAAGCTGCCACATG

TAACGCCCTTGCTGCTTCTGTGTGTGGGTGATTGGATGTGGAGGCGGGGCCCGTG

GAGAGCCCGTGCCCCTCCCCACCTCCCTGTGCCTGTATCTAATATATAAATA

TAGAGATGTGTATATGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

FIG.2B(iii)

Pro Trp Phe Tyr Arg Arg Leu Val Leu Ser Ser Val Leu Ser Ser Leu Leu Val Pro
CCG TGG TTC TAC CGG CGG TTA GTT CTC TCT TCT GTG TTG TCC TCC CTC CTC GTT CCC

Asp Arg Arg Gln Pro Ala Thr Arg Ala Ala Ala Arg Phe Leu Trp Glu Ala Gln
GAT CGC CGC CAG CCG GCT ACA CGG GCG GCG GCG CGG TTC CTG TGG GAA GCG CAG 111

His Lys Ser Ser Gly Asn Ala Lys Arg Arg Ala Gln Arg Gly Gly Gly Cys Ala Ala
CAC AAG TCG AGC GGT AAC GCG AAG CGT CGA GCC CAA CGC GGC GGA GGC TGT GCA GCC

Asn MET Ala Ala Ala Ala Ala Ala Gly Pro Glu MET Val Arg Gly Gln Val Phe
AAC ATG GCG GCG GCG GCG GCG GCG GGC CCG GAG ATG GTC CGC GGG CAG GTG TTC 222

* * I *
Asp Val Gly Pro Arg Tyr Thr Asn Leu Ser Tyr Ile Gly Glu Gly Ala Tyr Gly Met
GAC GTG GGG CCG CGC TAC ACT AAT CTC TCG TAC ATC GGA GAA GGC GCC TAC GGC ATG

* * II *
Val Cys Ser Ala Tyr Asp Asn Leu Asn Lys Val Arg Val Ala Ile Lys Lys Ile
GTT TGT TCT GCT TAT GAT AAT CTC AAC AAA GTT CGA GTT GCT ATC AAG AAA ATC 333

III *
Ser Pro Phe Glu His Gln Thr Tyr Cys Gln Arg Thr Leu Arg Glu Ile Lys Ile Leu
AGT CCT TTT GAG CAC CAG ACC TAC TGT CAG AGA ACC CTG AGA GAG ATA AAA ATC CTA

IV
Leu Arg Phe Arg His Glu Asn Ile Ile Gly Ile Asn Asp Ile Ile Arg Ala Pro
CTG CGC TTC AGA CAT GAG AAC ATC ATC GGC ATC AAT GAC ATC ATC CGG GCA CCA 444

V
Thr Ile Glu Gln Met Lys Asp Val Tyr Ile Val Gln Asp Leu Met Glu Thr Asp Leu
ACC ATT GAG CAG ATG AAA GAT GTA TAT ATA GTA CAG GAC CTC ATG GAG ACA GAT CTT

Tyr Lys Leu Leu Lys Thr Gln His Leu Ser Asn Asp His Ile Cys Tyr Phe Leu
TAC AAG CTC TTG AAG ACA CAG CAC CTC AGC AAT GAT CAT ATC TGC TAT TTT CTT 555

VI *
Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asn Val Leu His Arg Asp
TAT CAG ATC CTG AGA GGA TTA AAG TAT ATA CAT TCA GCT AAT GTT CTG CAC CGT GAC

* VII *
Leu Lys Pro Ser Asn Leu Leu Leu Asn Thr Thr Cys Asp Leu Lys Ile Cys Asp
CTC AAG CCT TCC AAC CTC CTG CTG AAC ACC ACT TGT GAT CTC AAG ATC TGT GAC 666

FIG.3A(i)

```
 *   *                                                          VIII
Phe Gly Leu Ala Arg Val Ala Asp Pro Asp His Asp His Thr Gly Phe Leu Thr Glu
TTT GGC CTT GCC CGT GTT GCA GAT CCA GAC CAT GAT CAT ACA GGG TTC TTG ACA GAG
                                *   *   *
Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu Ile Met Leu Asn Ser Lys Gly
TAT GTA GCC ACG CGT TGG TAC AGA GCT CCA GAA ATT ATG TTG AAT TCC AAG GGT  777
                     *           IX      *
Tyr Thr Lys Ser Ile Asp Ile Trp Ser Val Gly Cys Ile Leu Ala Glu Met Leu Ser
TAT ACC AAG TCC ATT GAT ATT TGG TCT GTG GGC TGC ATC CTG GCA GAG ATG CTA TCC
Asn Arg Pro Ile Phe Pro Gly Lys His Tyr Leu Asp Gln Leu Asn His Ile Leu
AAC AGG CCT ATC TTC CCA GGA AAG CAT TAC CTT GAC CAG CTG AAT CAC ATC CTG  888
                X
Gly Ile Leu Gly Ser Pro Ser Gln Glu Asp Leu Asn Cys Ile Ile Asn Leu Lys Ala
GGT ATT CTT GGA TCT CCA TCA CAG GAA GAT CTG AAT TGT ATA ATA AAT TTA AAA GCT
Arg Asn Tyr Leu Leu Ser Leu Pro His Lys Asn Lys Val Pro Trp Asn Arg Leu
AGA AAC TAT TTG CTT TCT CTC CCG CAC AAA AAT AAG GTG CCG TGG AAC AGG TTG  999
                                                                       XI
Phe Pro Asn Ala Asp Ser Lys Ala Leu Asp Leu Leu Asp Lys Met Leu Thr Phe Asn
TTC CCA AAC GCT GAC TCC AAA GCT CTG GAT TTA CTG GAT AAA ATG TTG ACA TTT AAC
             *
Pro His Lys Arg Ile Glu Val Glu Gln Ala Leu Ala His Pro Tyr Leu Glu Gln
CCT CAC AAG AGG ATT GAA GTT GAA CAG GCT CTG GCC CAC CCG TAC CTG GAG CAG  1110
Tyr Tyr Asp Pro Ser Asp Glu Pro Ile Ala Glu Ala Pro Phe Lys Phe Asp Met Glu
TAT TAT GAC CCA AGT GAT GAG CCC ATT GCT GAA GCA CCA TTC AAG TTT GAC ATG GAG
Leu Asp Asp Leu Pro Lys Glu Lys Leu Lys Glu Leu Ile Phe Glu Glu Thr Ala
CTG GAC GAC TTA CCT AAG GAG AAG CTC AAA GAA CTC ATT TTT GAA GAG ACT GCT  1221
Arg Phe Gln Pro Gly Tyr Arg Ser END
CGA TTC CAG CCA GGA TAC AGA TCT TAA ATTGGTCAGGACAAGGGCTCAGAGGACTGGACGCGTTCA
GATGTCGGTGTCCCCCAGTTCTTGACCCTGGTCCTGTCTCCAGCCCGTCTCAGCTTACCCACTCTTGACTC  1359
CTTTGAGCCGTTCCGAGGGGCAGTCTGGTCGTAGTGGCTTTTATACTTTCACGGAATTCTTCAGTCCAGAGAGTT
CTCCTGCACCAGGCCCTGCACAGTTGCACTCAG                                          1467
```

FIG.3A(ii)

AGACCTGCCGGGCGCATATTTATTCACAGTTTTGTCCCATGTTAAGTCGGTTA

GCATAGTGAATCTGAGTGCATAGTATGTCATTTCATTCCGTTGAGTTTCTCGA

GTGTTTTCTTTAAATGTCTGCAGAGTCGCTACCCTTCCTTGAACTATGAAGCAC 160

TGCAATCTTCTTAATTCTCAGTATGAAGAGAGATTTTTGAGCTTTAAGTCTGA

GGGGAACTCAGCAGGCCTGGTTGGCGTCTGCAATGAACATCAAGAAACCATCG

END Glu Ile Phe Pro

TGCTGTGGGAATGTGATCGTTTTTCTCCCTTTT TGA GAG ATC TTT CCT 314

Phe Asp Ala Ser Phe Leu Pro Cys Leu His Lys Phe Asn

TTT GAT GCC AGT TTT CTT CCT TGT TTA CAC AAG TTC AAC

Asn Leu Lys Gly Lys Gly Asn Cys Lys Gly Phe Lys MET

AAT TTG AAA GGA AAA GGC AAT TGT AAG GGT TTT AAA ATG

Ala Glu Lys Phe Glu Ser Leu Met Asn Ile His Gly Phe Asp

GCA GAG AAA TTT GAA AGT CTC ATG AAC ATT CAT GGC TTT GAT 434

*

Leu Gly Ser Arg Tyr Met Asp Leu Lys Pro Leu Gly Cys

CTG GGT TCC AGG TAC ATG GAC TTA AAA CCA TTG GGC TGT

* I * *

Gly Gly Asn Gly Leu Val Phe Ser Ala Val Asp Asn Asp

GGA GGC AAT GGC TTG GTT TTT TCT GCT GTA GAC AAT GAC

* II *

Cys Asp Lys Arg Val Ala Ile Lys Lys Ile Val Leu Thr Asp

TGT GAC AAA AGA GTA GCC ATC AAG AAA ATT GTC CTC ACC GAT 554

III *

Pro Gln Ser Val Lys His Ala Leu Arg Glu Ile Lys Ile

CCC CAG AGT GTC AAA CAT GCC CTC CGT GAA ATC AAA ATT

IV

Ile Arg Arg Leu Asp His Asp Asn Ile Val Lys Val Phe

ATT AGA AGA CTT GAC CAC GAT AAC ATT GTG AAA GTG TTT

Glu Ile Leu Gly Pro Ser Gly Ser Gln Leu Thr Asp Asp Val

GAA ATT CTT GGT CCC AGT GGA AGC CAG CTG ACA GAC GAT GTG 674

Gly Ser Leu Thr Glu Leu Asn Ser Val Tyr Ile Val Gln

GGC TCT CTA ACA GAG CTG AAT AGC GTC TAC ATT GTT CAG

V

Glu Tyr Met Glu Thr Asp Leu Ala Asn Val Leu Glu Gln

GAG TAC ATG GAG ACA GAC TTG GCG AAC GTG CTG GAG CAG

Gly Pro Leu Leu Glu Glu His Ala Arg Leu Phe Met Tyr Gln

GGC CCT TTA CTG GAG GAG CAT GCC AGG CTC TTC ATG TAC CAG 794

FIG.3B(i)

```
                                      VI
Leu Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asn Val
CTG CTG CGT GGG CTC AAG TAC ATC CAC TCT GCA AAC GTG
            *               *
 Leu His Arg Asp Leu Lys Pro Ala Asn Leu Phe Ile Asn
CTG CAC AGG GAT CTC AAG CCG GCC AAC CTT TTC ATT AAC
                     VII             *   *   *
Thr Glu Asp Leu Val Leu Lys Ile Gly Asp Phe Gly Leu Ala
ACT GAA GAC TTG GTG CTG AAG ATT GGT GAC TTT GGC CTG GCC    914
                                                VIII
Arg Ile Met Asp Pro His Tyr Ser His Lys Gly His Leu
CGG ATC ATG GAT CCT CAT TAT TCC CAT AAG GGT CAT CTT
                                         *   *   #
Ser Glu Gly Leu Val Thr Lys Trp Tyr Arg Ser Pro Arg
TCT GAA GGA TTG GTT ACC AAA TGG TAC AGA TCT CCA CGG
                                                 *
Leu Leu Leu Ser Pro Asn Asn Tyr Thr Lys Ala Ile Asp Met
CTT TTA CTT TCT CCT AAT AAC TAT ACT AAA GCC ATT GAC ATG    1034
        IX   *
Trp Ala Ala Gly Cys Ile Phe Ala Glu Met Leu Thr Gly
TGG GCT GCA GGC TGC ATC TTT GCT GAA ATG CTG ACT GGT
Lys Thr Leu Phe Ala Gly Ala His Glu Leu Glu Gln Met
AAA ACC CTC TTT GCA GGT GCA CAT GAA CTT GAA CAG ATG
                                X
Gln Leu Ile Leu Glu Ser Ile Pro Val Val His Glu Glu Asp
CAG CTG ATC TTG GAG TCT ATC CCT GTT GTG CAC GAG GAA GAT    1154
Arg Gln Glu Leu Leu Ser Val Ile Pro Val Tyr Ile Arg
CGG CAG GAG CTT CTC AGC GTG ATT CCA GTT TAC ATT AGA
Asn Asp Met Thr Glu Pro His Lys Pro Leu Thr Gln Leu
AAC GAC ATG ACT GAG CCA CAC AAA CCG CTG ACT CAG CTG
Leu Pro Gly Ile Ser Arg Glu Ala Leu Asp Phe Leu Glu Gln
CTT CCG GGG ATT AGT CGG GAA GCA CTG GAT TTC CTG GAA CAG    1274
                   XI           *
Ile Leu Thr Phe Ser Pro Met Asp Arg Leu Thr Ala Glu
ATT CTG ACG TTC AGT CCC ATG GAC CGG CTG ACA GCC GAG
Glu Ala Leu Ser His Pro Tyr Met Ser Ile Tyr Ser Phe
GAA GCA CTT TCC CAT CCT TAC ATG AGC ATC TAC TCT TTC
Pro Thr Asp Glu Pro Ile Ser Ser His Pro Phe His Ile Glu
CCA ACG GAC GAG CCT ATT TCC AGC CAT CCT TTC CAC ATA GAA    1394
```

FIG.3B(ii)

Asp Glu Val Asp Asp Ile Leu Leu Met Asp Glu Thr His
GAC GAA GTG GAC GAC ATT TTG CTA ATG GAT GAA ACA CAC

Ser His Ile Tyr Asn Trp Glu Arg Tyr His Asp Cys Gln
AGT CAC ATT TAT AAC TGG GAA AGG TAC CAC GAT TGT CAG

Phe Ser Glu His Asp Trp Pro Ile His Asn Asn Phe Asp Ile
TTC TCG GAG CAT GAC TGG CCT ATT CAT AAC AAC TTT GAT ATC 1514

Asp Glu Val Gln Leu Asp Pro Arg Ala Leu Ser Asp Val
GAT GAG GTT CAG CTT GAC CCG AGA GCT CTG TCT GAT GTC

Thr Asp Glu Glu Glu Val Gln Val Asp Pro Arg Lys Tyr
ACC GAT GAA GAA GAA GTT CAA GTT GAT CCT CGA AAG TAC

Leu Asp Gly Asp Arg Glu Lys Tyr Leu Glu Asp Pro Ala Phe
TTG GAT GGA GAC CGA GAG AAG TAT CTG GAG GAT CCC GCC TTC 1634

Asp Thr Ser Tyr Ser Ala Glu Pro Cys Trp Gln Tyr Pro
GAC ACC AGC TAC TCT GCT GAG CCT TGC TGG CAG TAC CCA

Asp His His Glu Asn Lys Tyr Cys Asp Leu Glu Cys Ser
GAT CAC CAC GAG AAC AAG TAC TGT GAT CTG GAG TGT AGC

His Thr Cys Asn Tyr Lys Thr Arg Ser Pro Ser Tyr Leu Asp
CAC ACC TGT AAC TAC AAA ACA AGG TCG CCA TCA TAC TTA GAT 1754

Asn Leu Val Trp Arg Glu Ser Glu Val Asn His Tyr Tyr
AAC CTG GTG TGG AGG GAG AGC GAG GTT AAC CAT TAC TAT

Glu Pro Lys Leu Ile Ile Asp Leu Ser Asn Trp Lys Glu
GAG CCC AAG CTT ATT ATA GAT CTT TCC AAC TGG AAA GAG

Gln Ser Lys Asp Lys Ser Asp Lys Arg Gly Lys Ser Lys Cys
CAA AGT AAG GAC AAA TCC GAC AAG AGA GGC AAG TCC AAG TGT 1874

Glu Arg Asn Gly Leu Val Lys Arg Arg Leu Arg Leu Arg
GAG AGG AAC GGG TTG GTC AAG CGC AGA TTG CGC TTG AGG

Lys Arg Pro Ser Ser Trp Leu Arg Gly Arg Gly Ala Lys
AAG CGT CCC AGC AGC TGG CTG AGA GGG AGA GGG GCC AAG

Ala Leu Thr Leu Met Pro Ser Ser Gln Ala Pro Phe Ser Ser
GCT TTG ACT TTG ATG CCT TCA TCG CAG GCA CCG TTC AGC TCA 1994

FIG.3B(iii)

```
Val Pro Ser Val Ser Leu Leu Thr END
GTG CCC AGC GTG AGT CTG CTG ACG TAG TTGACAAGTTAAACGAC
TTGAATAGCTCAGTGTCCCAGCTAGAAATGAAAAGCCTGATATCCAAGTCAGT
CAGCCGAGAAAAGCAAGAAAAGGGAAGGGCTAACTGGCCCAGCTGGGAGCCTT   2145
GTACCAGCCCTCCTGGGAGAGCCAGTTTGTGAGTGGCGGGGAGGAGTGCTTCC
TTATCAGTCAGTTTTGTTGTGAGGTCAGGAAGGACGAACACGTGGAGAAGGAG
AACACTTACACCAGCTATTTGGACAAGTTTTTTAGCAGGAAGGAGGATTCTGAA  2305
ATGCTAGAAACTGAGCCAGTGGAAGAAGGGAAGCGTGGGGAGAGAGGCCGTGA
GGCAGGGCTTCTGAGCAGCGGTGGGGAGTTTCTCCTGAGCAGGCAGCTAGAGT
CCATAGGCACCCCGCAGTTCCACAGTCCAGGGGGATCCCCACTCAAGTCCATCC  2465
AGGCCACGTTAACACCTTCCGCTATGAAATCTTCCCCTCAAATCCCTCACAAG
ACATACAGCAACATTCTGAAACATCTGAACTAAACACTCAGCAGACACTTCTT
TTGTTCTTCATGAAATGTGTTGTGTCTTTTTTATCACTAATGTTTTAAGTCATT  2625
TTTTTTTTACTTGAATCAGAAGGTGTCATTAATTTGCAAGGATTTTTCTTGGT
TCTCAGTTTGTAAAACACAGAGTTTTTTCTACATGTGAGTTAGTTTTCATTTG
AACTGGCATGTCGTTTGCACACACACAAAGAATAGAGCAAAACAATGCAGTGCA  2785
GGAGGAGACAAGATGCGCTAGGATGGACAGACATTCTCACAGACCAGTGACCT
GCTTACAGGAAACAAAACCTTGCCTTGAAACTTACACAGTGAGACTGTACATA
ATTGCATGAAAAGATCTATTTTTTTCCTGAAACATTTTTCATTCATTAGTATTT  2945
TCAAGTTTTTCATACTGTACACATTTCTTAAGACACATGATACCAGCAGCAAC
TGAAAACGAATGCCGAATTTGGTACACATGTGTTATCTACCTCAAGGTAACAA
AAGTATGCGGGCGAAACCTAACCCACCCATAGTCGTCCGCGGCATATGCACTTG  3105
TATCTAGCCAGCGTTGGCCGCAGTAACCAATGAGACTCGTCCGCCATTTATCA
ATGTCCTGGTGTTCATCCTTTACAGTGAAGTGTTAGATACATCACATCTTATT
TATTTTTAGCAAATCAGTATATTTTCTGTATTTAATTATAAAAGGTAACTTAGT  3265
TTAAGTTTATTTGCAACTGCCCTTCTTCCCGTTTGGCACTATGGTTTGTTGCC
TGCCGAGCTGATCTGAGAAGTCAGCTTGTCCCGAGGCTGTCCATGTACGTTAA
GTAAAGTGCTCACTGTGTATAGGAATCTGTATTTTGGAGGTGCTTGATCTATCT  3420
ACAAAGAAAAAAATTAGGAATTTATTATAAAATGCTCCTAGAAGTCTTAATGG
TGTTTATTTTTTAAAACCTTGTAATGTTAGACTTGTGTGCATGGAAGTGATTA
AGGTACATCATTATTGTAGTTTGAACATTGTACATGATAAGCCTTCCCCCACCC  3585
CCGTTTTTACTGTATGTTTTTATTGAATGATCTATTCCCCATCCCTAGGCAAG
CATGAATAAAATTAGGTTAAATGTAAAAAAAAA                       3671
```

FIG.3B(iv)

```
        Ala Pro Gly Gly Gly Gly Gly Glu Pro Arg Gly Thr Ala Gly Val Val
ERK1    GCT CCG GGG GGC GGG GGC GGG GAG CCC AGG GGA ACT GCT GGG GTC GTC
                    Asp
ERK1Ψ   ... ... ... .AT ... ... ... ... ... ... ... ... ... ... ... ...

        Pro Val Val Pro Gly Glu Val Glu Val Val Lys Gly Gln Pro Phe Asp Val
        CCG GTG GTC CCC GGG GAG GTG GAG GTG GTG AAG GGG CAG CCA TTC GAC GTG  99
        Leu             Arg             --- --- ---
        .T. ... ... ... C.. ... ... ... --- --- --- ... ... ... ... ... ...

                                            *       # I         #
        Gly Pro Arg Tyr Thr Gln Leu Gln Tyr Ile Gly Glu Gly Ala Tyr Gly
ERK1    GGC CCA CGC TAC ACG CAG CTG CAG TAC ATC GGC GAG GGC GCG TAC GGC
                Tyr                                     Asp     --- Asp
ERK1Ψ   ... ... T.T ... ..A ... ... ... ... ... ... ..A .AT ... --- .A.

            *                                               * II    *
        Met Val Ser Ser Ala Tyr Asp His Val Arg Lys Thr Arg Val Ala Ile Lys
        ATG GTC AGC TCA GCA TAT GAC CAC GTG CGC AAG ACC AGA GTG GCT ATC AAG  198
        ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ...
```

FIG.3C(i)

```
                                                                    III
       Lys Ile Ser Pro Phe Glu His Gln Thr Tyr Cys Gln Arg Thr Leu Arg
ERK1   AAG ATC AGC CCC TTC GAG CAT CAA ACC TAC TGT CAG CGC ACG CTG AGA
                                                       Pro
ERK1   ... ..T ... ... ... ... ... ... ... ... ... ... .C. ... T.. ...

       Glu Ile Gln Ile Leu Leu Gly Phe Arg His Glu Asn Val Ile Gly Ile Arg
       GAA ATC CAG ATC TTG CTC GGA TTC CGC CAT GAG AAT GTC ATA GGC ATC CGA  297
           Thr                 Arg
       ... .C. ... ..A ... ..G C.. ... ... ... ... ... ... ... ... ... ...

       Asp Ile Leu Arg Ala Pro Thr Leu Glu Ala Met Arg Asp Val Tyr Ile
ERK1   GAC ATC CTC AGA GCA CCC ACC CTG GAA GCC ATG AGA GAT GTT TAC ATT

ERK1Ψ  ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ...
                            V
       Val Gln Asp Leu Met Glu Thr Asp Leu Tyr Lys Leu Leu Lys Ser Gln Gln
       GTT CAG GAC CTC ATG GAG ACG GAC CTG TAC AAG CTG CTA AAG AGC CAG CAG  396
                           Asp
       ... ... ... ... ... ..T ..A ... ... ... ... ..T ... ... ..A ...
```

FIG.3C(ii)

```
                                      Leu Ser Asn Asp His Ile Cys Tyr Phe Leu Tyr Gln Ile Leu Arg Gly
ERK1    CTG AGC AAT GAC CAC ATC TGC TAC TTC CTC TAC CAG ATC CTC CGG GGC
                                                                 Phe
ERK1Ψ   ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ...

                         VI                                    *
        Leu Lys Tyr Ile His Ser Ala Asn Val Leu His Arg Asp Leu Lys Pro Ser
        CTC AAG TAC ATA CAC TCG GCC AAT GTG CTG CAC CGG GAC CTG AAG CCC TCC  495
        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

         *                                 VII             *   *   *
        Asn Leu Leu Ile Asn Thr Thr Cys Asp Leu Lys Ile Cys Asp Phe Gly Leu Ala
ERK1    AAT CTG CTT ATC AAC ACC ACC TGC GAC CTT AAG ATC TGT GAT TTT GGC CTT GCC
                                    END
ERK1Ψ   ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ..G ...
```

FIG.3C(iii)

```
                                          I                                II
ERK1          EPRGTAGVVPVVPGEVEVVKGQPFDVGPRYTQLQYIGEGAYGMVSSAYDHVRKTRVAIKKISPFE
ERK2                   MAAAAAAGP.M.R..V........N.S..........C./..NLN..V..........
ERK3                       MAEKFESLMNIHG..L.S..MD.KPL.C.GN.L.F..V.NDCDK.......VLTD
FUS3                                MPKRIVYNISSDFQLKSLL......V.C..THKPTGEI......E..D
KSS1                                MARTIT..IPSQ.KLVDL.......T.C..IHKPSGIK......Q..S
hCDC2                                            MED..KIEK....T..V.YKGRHKTTGQV..M...RLES

                             VI                           VII
ERK1    LSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLINTT-CDLKICDFGLARIADPEHD-------HTGF
ERK2    ..................................L...-............V...D..-------....
ERKS    .LEE.ARL.M..L..................A..F...EDLV...G.......M..HYS-------.K.H
FUS3    ..D...Q..I..T..AV.VL.GS..I............SN-....V........I.ESAADNSEPTGQQSG
KSS1    ..D..VQ..T.....A..S....Q.I...I......L.SN-....V.......CLASSS.---SRETLV..
hCDC2   MDSSLVKSY.....Q.IVFC..RR........Q....DDK-GTI.LA......AFG----------IPIRV

                                                        XI
ERK1    IINMKARNYLQSLPSKTKVAWAKLFPKSD--SKALDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDEP
ERK2    ...L......L...H.N..P.NR...NA.--........K......H...E..Q.............S...
ERK3    LLSV-IPV.IRNDMTEPHKPLTQ.L.GIS--RE...F.EQI...S.MD.L.A....S...MSI.SF.....
FUS3    .ESPR..E.IK...MYPAAPLE.M..RVN--P.GI...Q...V.D.A....AK...E....QT.H..N...
KSS1    .KSKR.KE.IAN..MRPPLP.ETVWS.T.LNPDMI....K..Q...D...SAA...R....AM.H..S...
hCDC2   VESL--QD.KNTF.KWKPGSL.SHVKNL.--ENG....SK..IYD.A...SGKM..N...FNDLDNQIKKM

ERK3    SDVTDEEEVQVDPRKYLDGDREKYLEDPAFDTSYSAEPCWQYPDHHENKYCDLECSHTCNYKTRSPSYLDN

ERK3    SQAPFSSVPSVSLLT  543
```

FIG. 4A(i)

```
   III                 IV                      V
HQT-YCQRTLREIQILLGFR-HENVIGIRDILRAPTL-----EAMRDVYIVQDLMETDLYKLLKSQ-----Q  125
...-.........K...R..-...I...N..I....I-----.Q.K..................T.-----H  118
P.S--VKHA....K.IRRLD-.D.IVKVFE..GPSQSGLTDDTELNS.....EY.....ANV.EQG-----P  123
KPL-FAL......K..KH.K-...I.T.FN.Q.PDSF-----.NFNE...I.E..Q...HRVIST.-----M  108
KKL-FVT..I...KL.RY.HE...I.S.L.KV.PVSI-----DKLNA..L.EE......Q.VINN.NSGFST  114
EEEGVPSTAI...SL.KEL.-.P.IVSLQ.V.MQD----------SRL.LIFEFLSM..K.Y.D.IPPGQ-Y   99

 VIII                      IX                           X
LTEYVATRWYRAPEIMLNSKGYTKSIDIWSVGCILAEMLSNRPIFPGKHYLDQLNHILGILGSP-SQEDLNC  259
................................................................-.......  252
.S.GLV.K...S.RLL.SPNN...A..M.AA...F....TGKTL.A.A.E.E.MQL..ESIPVV-HE..RQE  258
M.............V..T.AK.SRAM.V..C......LFLR......RD.RH..LL.F..I.T.H.DN..R.  250
M.................TFQE..TAM....C.......V.GK.L...RD.HH..WL..EV..T.-.F..F.Q  25.
Y.H..V.L...S..VL.G.AR.STPV....I.T.F..LATKK.L.H.DSEI...FR.FRA..T.-NN.VWPE  230

VAEE------------PFTFDMELDD-LPKERLKELIFQETARFQPGAPEAP                      367
I..A------------..K.......-....K......E........YRS                        358
ISSH------------..HIED.V..I.LMDETHSH.Y-NWE.YHDCQFSEHDWPIHNNFDIDEVQLDPRAL  387
EG.PIPPSF--------.E..HHKEA-.TTKD..K..WN.IFS                               353
EYPPLNLDDEFWKLDNKIMRPE.EEE-V.I.M..DMLYD.LMKTME                            368
                                                                          297

LVWRESEVNHYYEPKLIIDLSNWKEQSKDKSDKRGKSKCERNGLVKRRLRLRKRPSSWLRGRGAKALTLMPS  528
```

FIG.4A(ii)

	ERK1	ERK2	ERK3	FUS3	KSS1	CDC2
ERK1	100	90	50	56	56	41
ERK2		100	51	55	57	41
ERK3			100	36	37	26
FUS3				100	57	30
KSS1					100	31
CDC2						100

FIG.4B

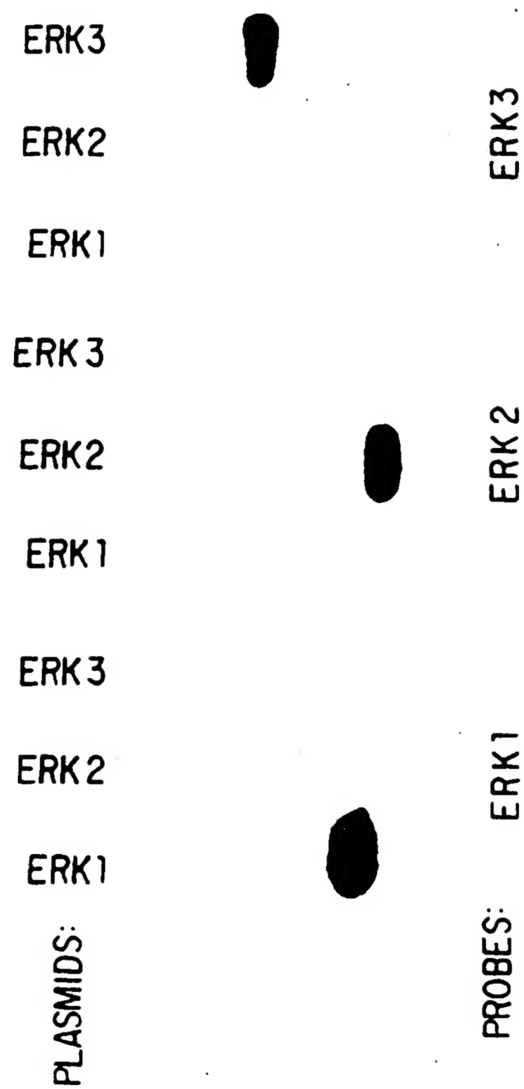
PLASMIDS:
ERK1
ERK2
ERK3
ERK1
ERK2
ERK3
ERK1
ERK2
ERK3
PROBES:
ERK1
ERK2
ERK3

FIG. 5A

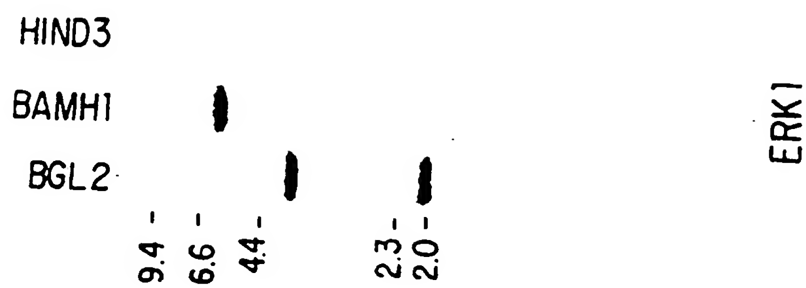
HIND3
BAMH1
BGL2
9.4
6.6
4.4
2.3
2.0
ERK1

FIG. 5C

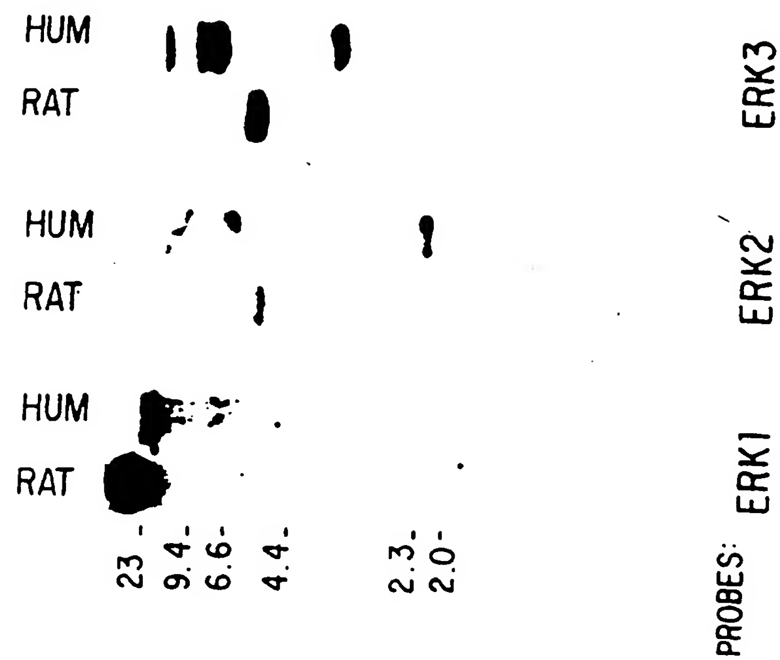
HUM
RAT
HUM
RAT
HUM
RAT
23
9.4
6.6
4.4
2.3
2.0
PROBES:
ERK1
ERK2
ERK3

FIG. 5B

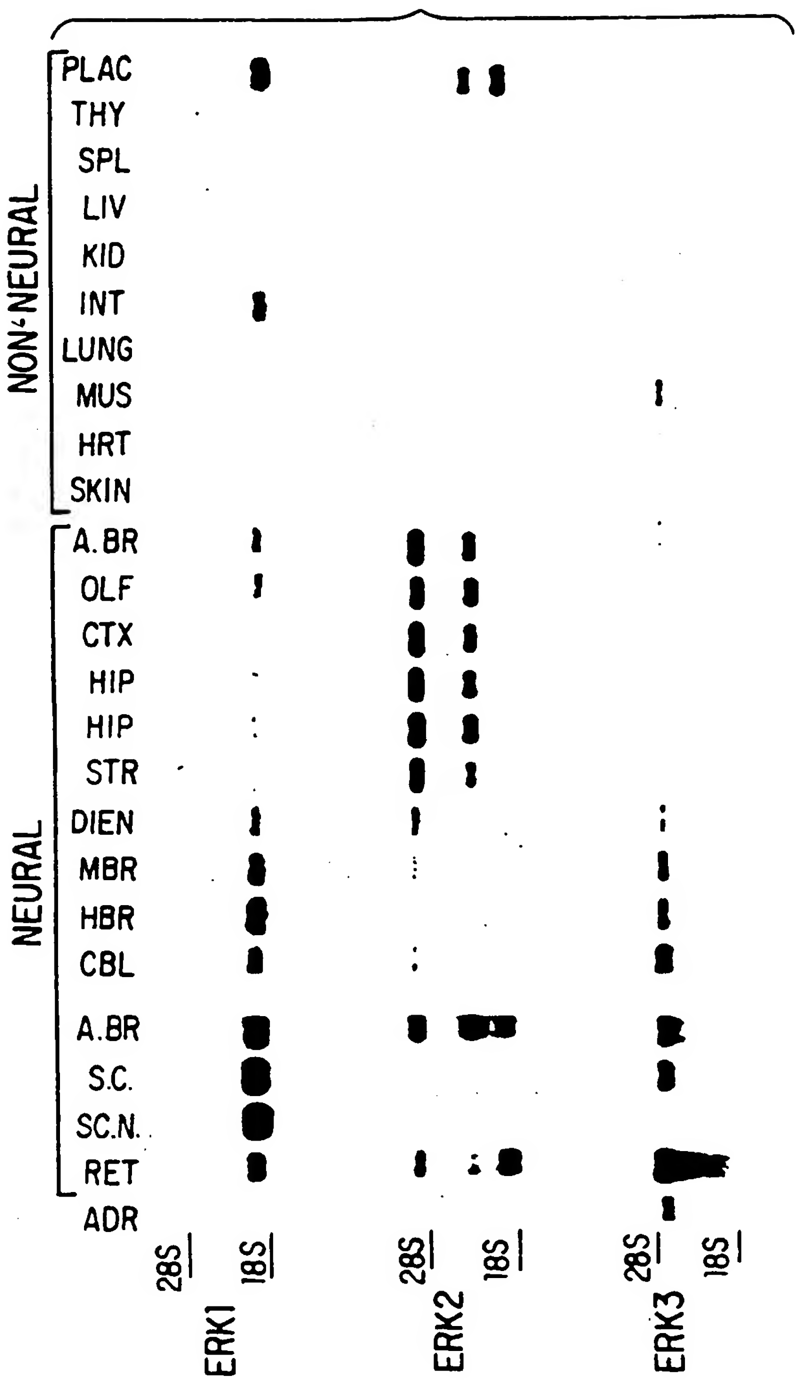
NON-NEURAL
PLAC
THY
SPL
LIV
KID
INT
LUNG
MUS
HRT
SKIN
NEURAL
A.BR
OLF
CTX
HIP
HIP
STR
DIEN
MBR
HBR
CBL
A.BR
S.C.
SC.N.
RET
ADR
28S
18S
ERK1
28S
18S
ERK2
28S
18S
ERK3

FIG. 6A

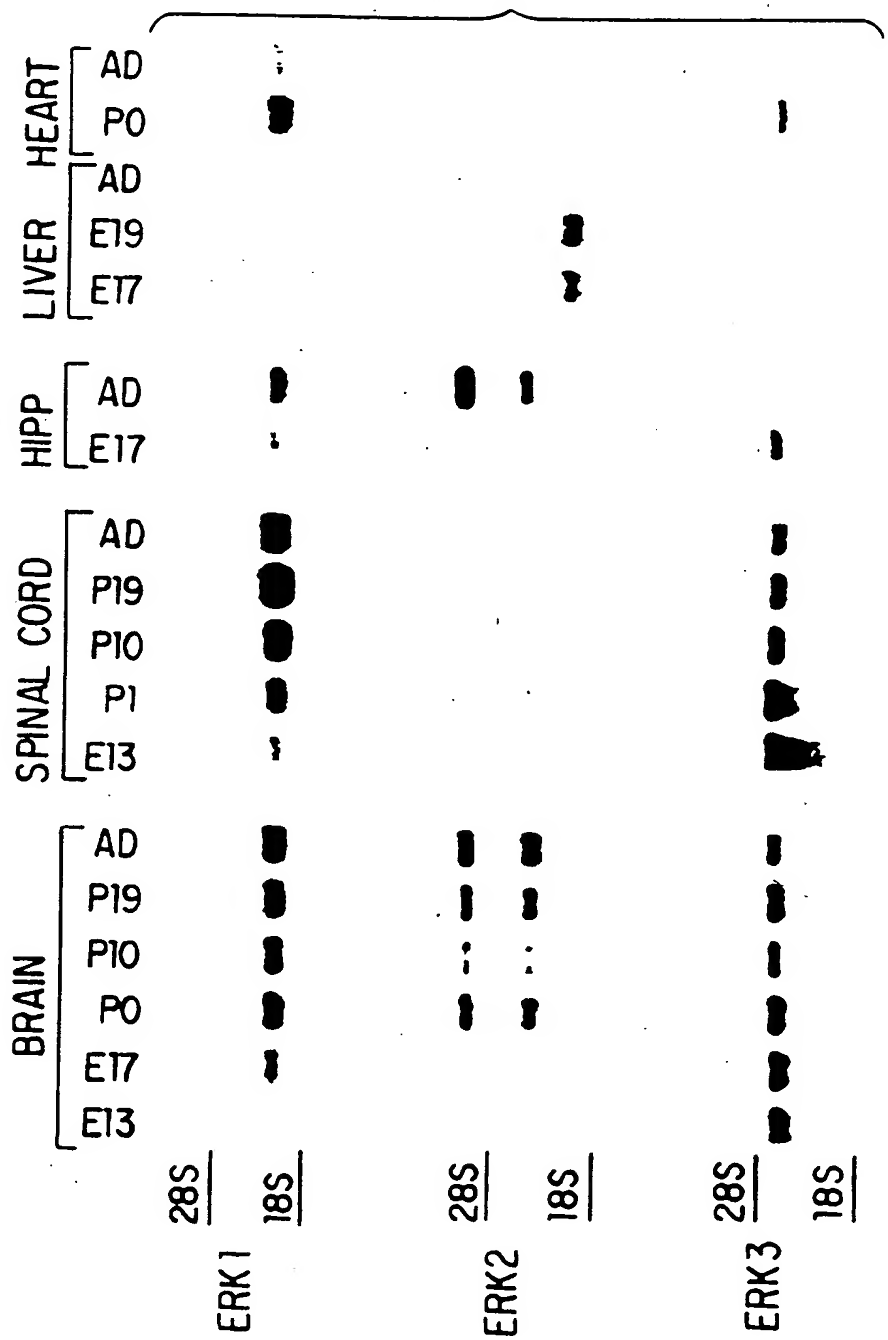
HEART
AD
P0
LIVER
AD
E19
E17
HIPP
AD
E17
SPINAL CORD
AD
P19
P10
P1
E13
BRAIN
AD
P19
P10
P0
E17
E13
28S
18S
28S
18S
28S
18S
ERK1
ERK2
ERK3

FIG. 6B

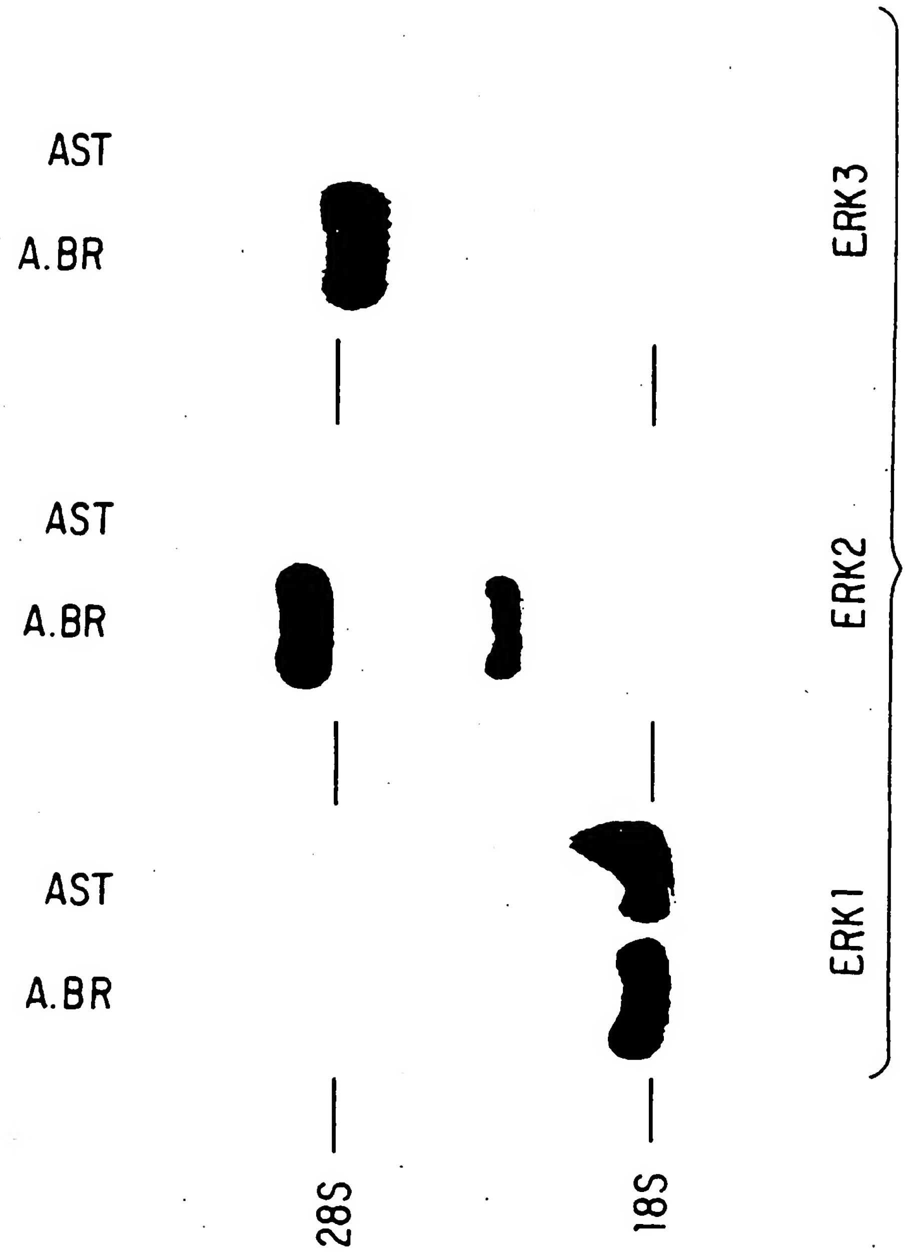

FIG. 6C

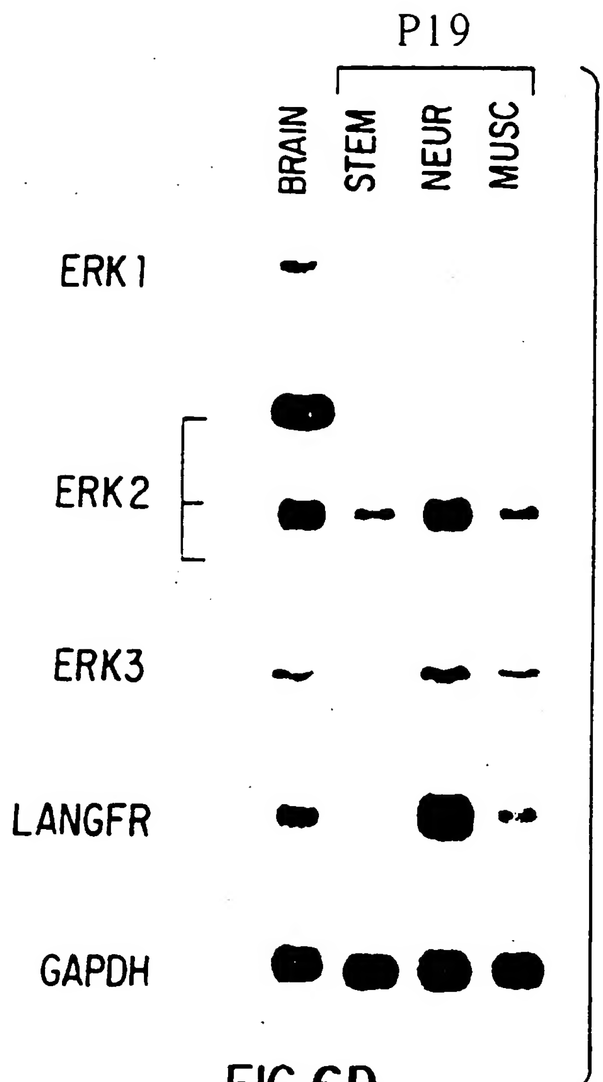

FIG.6D

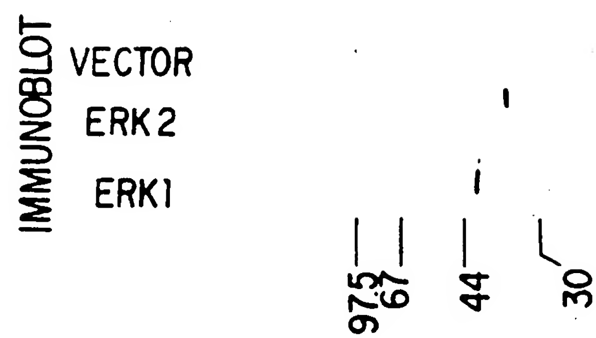
IMMUNOBLOT
VECTOR
ERK2
ERK1
97.5
67
44
30

FIG. 7B

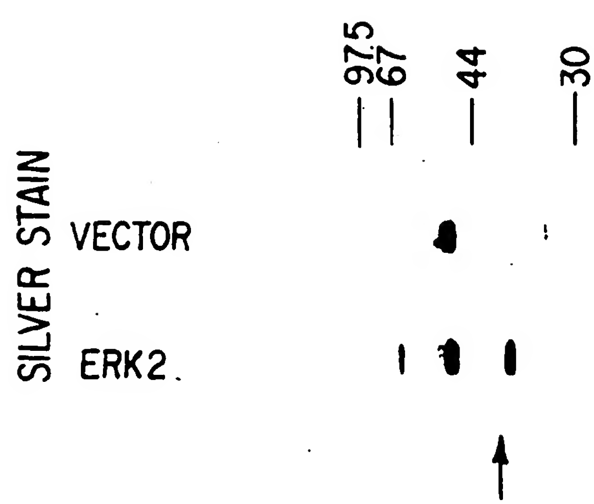
SILVER STAIN
VECTOR
ERK2
97.5
67
44
30

FIG. 7A

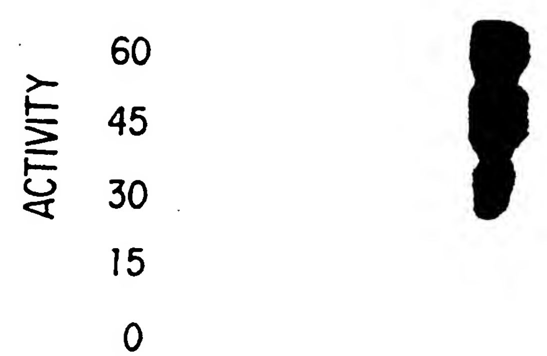
ACTIVITY
60
45
30
15
0

FIG. 7D

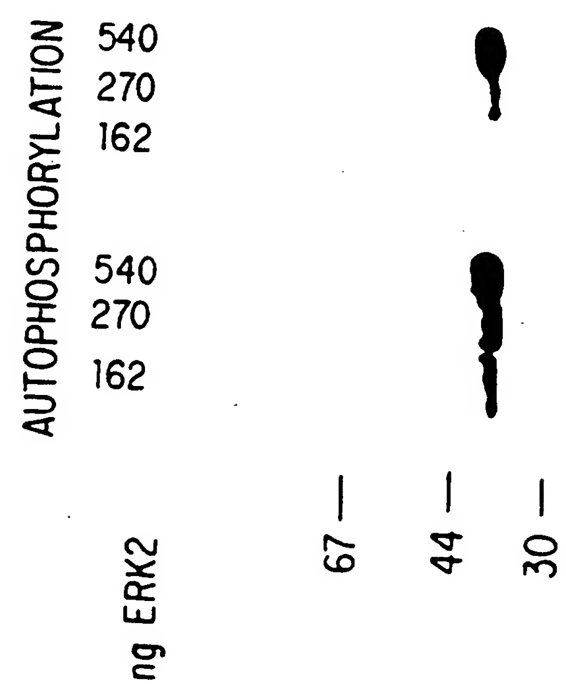
AUTOPHOSPHORYLATION
540
270
162
540
270
162
ng ERK2
67
44
30

FIG. 7C

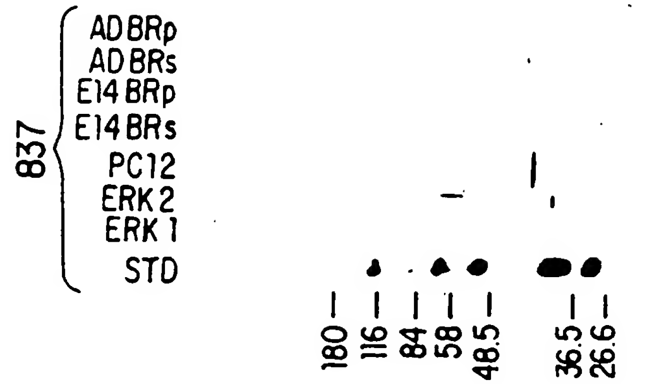

FIG. 8C

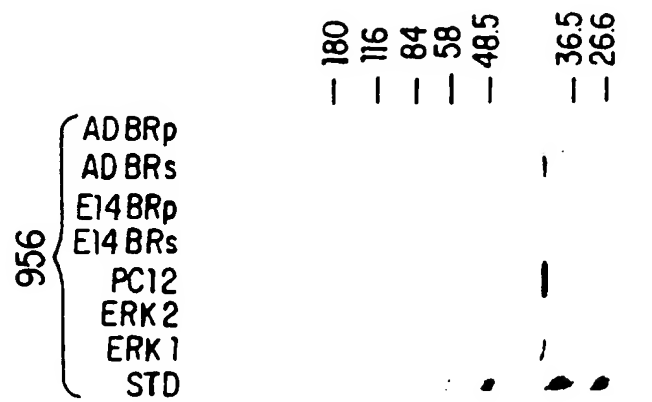

FIG. 8B

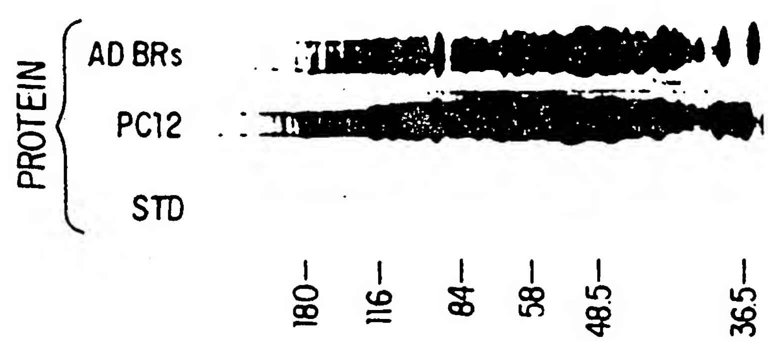

FIG. 8A

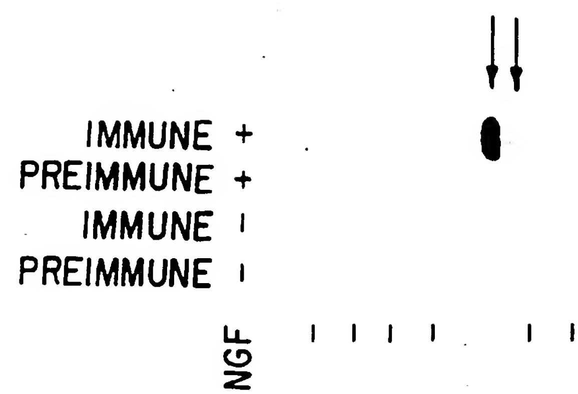
IMMUNE +
PREIMMUNE +
IMMUNE -
PREIMMUNE -
NGF

FIG. 9B

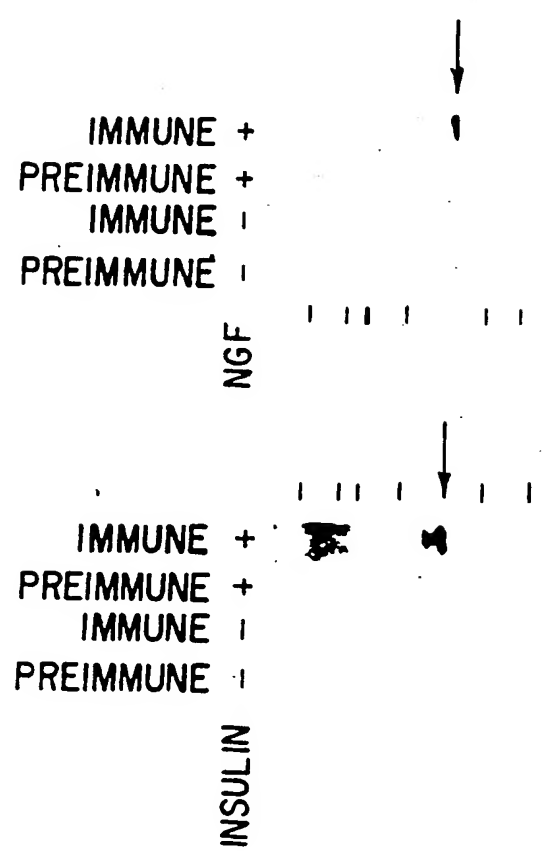
IMMUNE +
PREIMMUNE +
IMMUNE -
PREIMMUNE -
NGF
IMMUNE +
PREIMMUNE +
IMMUNE -
PREIMMUNE -
INSULIN

FIG. 9A

FIG. 9C

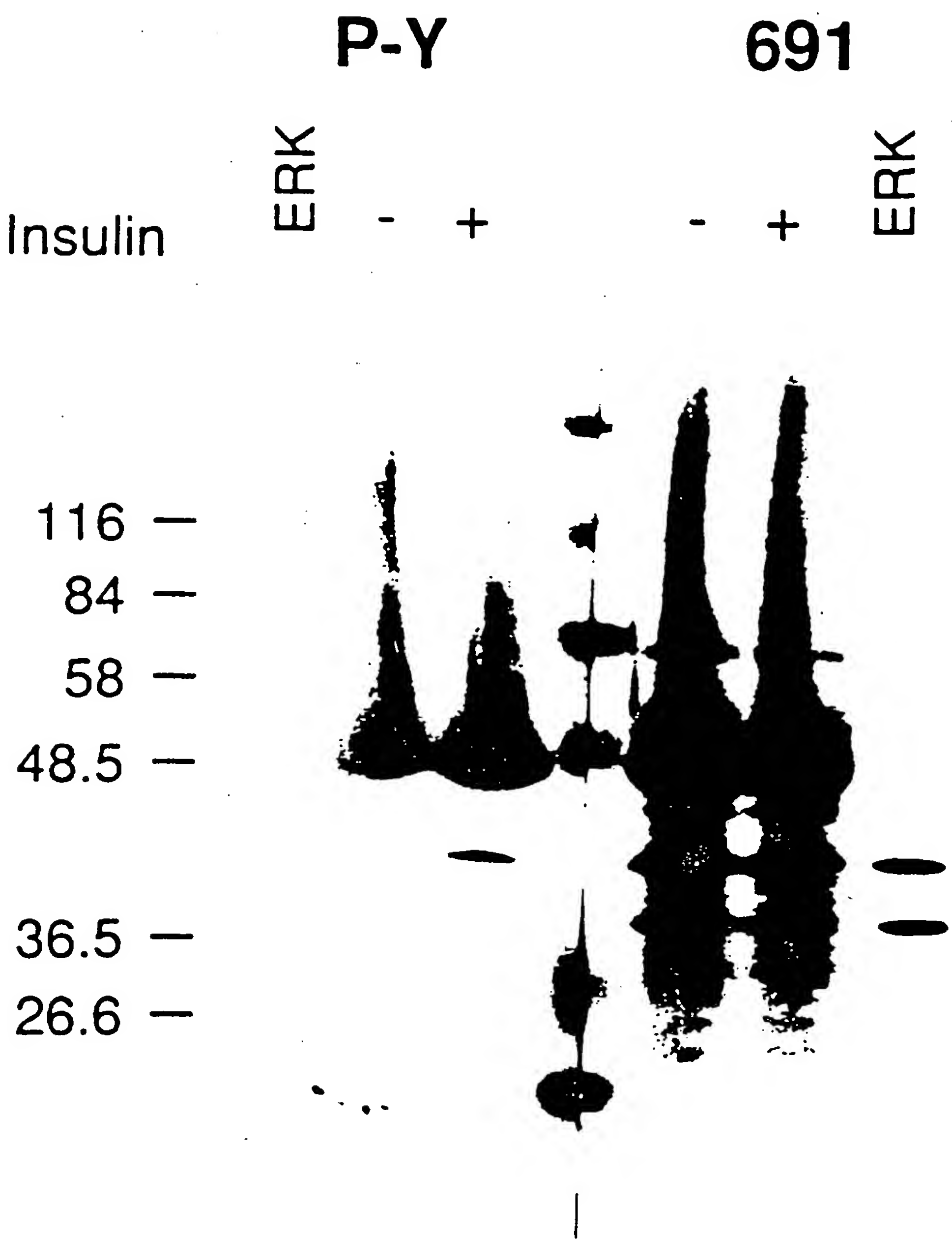

FIG. 10

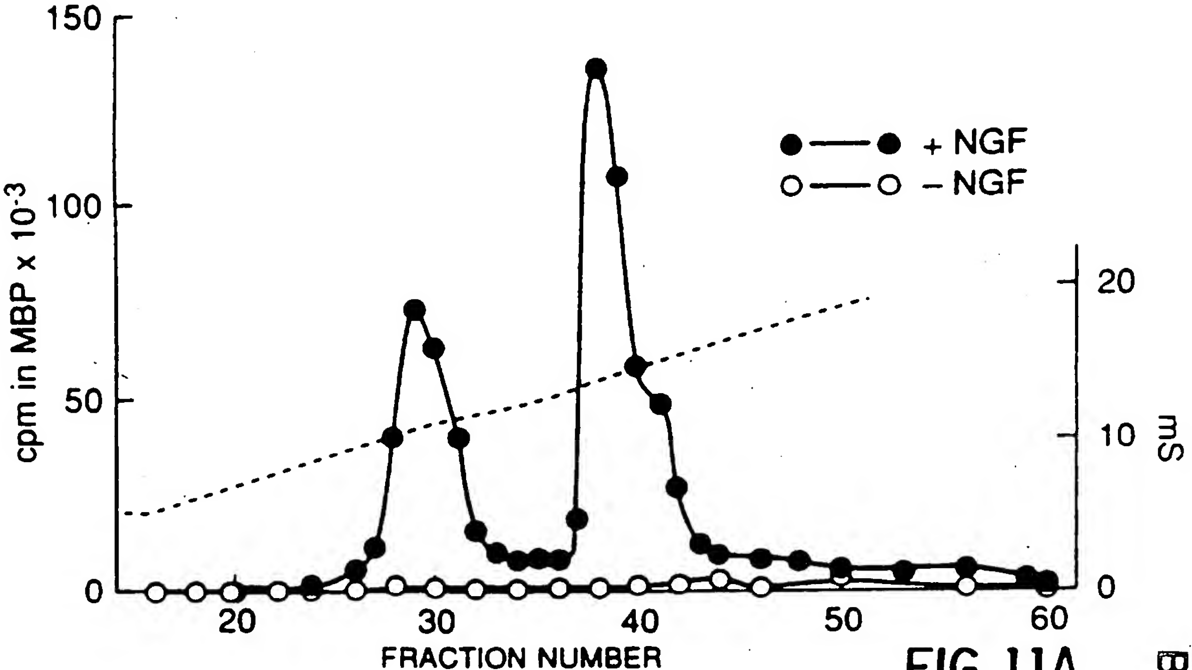

FIG. 11A

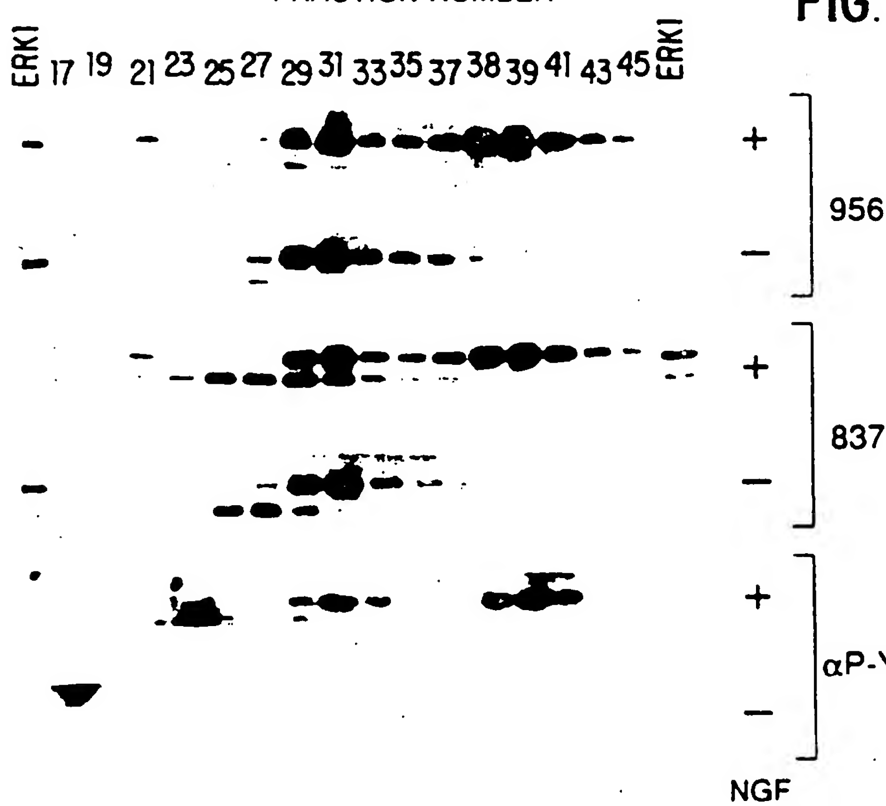

FIG. 11B

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INTERNATIONAL SEARCH REPORT

International Application No. PCT?US91/03894

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC
I.P.C. (5): C12Q 1/68, C07H 15/12; C07K 3/00; C12N 15/00
U.S. CL: 435/6; 536/27;935/77,78;530/350

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US. CL.	435/6,536/27;935/77,78; 530/350

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

GENBANK, EMBL

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
Y,P	Science. Vol. 249. issued July 1990. Boulton et al., "An Insulin-Stimulated Protein Kinase Similar to Yeast Kinases involved in Cell Cycle Control", pages 64-67. see especially Figs. 1 and 3.	1-45
X Y	Cell. vol. 58, issued 22 September 1989, Courchesne et al.. "A Putative Protein Kinase overcomes Pheromone-Induce Arrest of Cell Cycling in S. cerevisiae" page 1107-1119. see especially Figs. 293.	1-6,10, 17-21,28-31,34 7-9,11-16 22-28,32-33,35-45

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 September 1991	28 OCT 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Mindy B. Fleisher Mindy B. Fleisher TF

Form PCT/ISA/210 (second sheet) (Rev.11-87)

detecting changes in the levels of MAP2 protein kinase activity, wherein an increase in activity is indicative of the presence of insulin-like activity. The present invention therefore provides a powerful method for identifying compounds that may be useful in the treatment of diabetes. The present invention also provides for analogous methods which screen for the activity of other hormones or cellular factors. In additional embodiments of the invention, it may be desirable, in the above-mentioned screening methods, to utilize cell lines which comprises a recombinant nucleic acid molecule encoding a mammalian MAP2 kinase, including, but not limited to, recombinant nucleic acid molecules comprising sequences substantially as depicted in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5). Such cell lines may preferably express elevated levels of MAP2 kinase, and would therefore provide a more sensitive assay for MAP2 kinase activation. The present invention also provides for similar methods, in which cells utilized for screening comprise a recombinant nucleic acid sequence homologous to the sequence substantially as depicted in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5) or a portion thereof. The methods of the invention may be used to identify compounds that may be effective in the treatment of peripheral neuropathies or which may promote nerve regeneration. Furthermore, because NGF-responsive cholinergic neurons of the basal forebrain nucleic are consistently affected in early stages of Alzheimer's disease, the methods of the present invention may be particularly useful in identifying compounds with NGF-like activity which may be effective in the treatment of Alzheimer's disease. In addition, such methods may enable the identification of molecules capable of bypassing the hormone/receptor interaction. It may be clinically useful to inhibit the activity of MAP2 kinase in an organism,

using, for example, small molecules such as purine analogues.

In further embodiments of the present invention, recombinant MAP2 kinase may be used to identify other molecules, such as kinases related to cellular factor or hormone action. For example, recombinant MAP2 kinase could be used to identify additional kinases by affinity purification, wherein a MAP2 kinase may be used to adhere to other kinases which participate in a MAP2 associated phosphorylation cascade. Sequenced portions of the NGF receptor are likely to be physically associated with an as yet unidentified protein kinase. Recombinant MAP2 kinase may be useful in studying such interactions.

In another embodiment, detecting a change in a MAP2 protein kinase activity resulting from culturing cells in the presence of a compound known to or suspected to affect MAP2 protein kinase activity, can be used to detect the presence or measure the amount of such a compound and its ability to modulate MAP2 kinase activity levels. Such an effect on MAP2 kinase activity can occur directly or indirectly (e.g. through a signal transduction pathway). In a specific example of such an embodiment, the presence of a neurotrophin molecule (including but not limited to NGF, brain derived neurotrophic factor, neurotrophin-3 (NT-3) and other members of the NGF/BDNF/NT-3 family of molecules) can be detected by detecting an increase in the activity of a MAP2 protein kinase upon culturing the cells in the presence of a sample suspected of containing such a neurotrophin molecule. The cells which are cultured in such assays should express receptors for the neurotrophin molecule being detected, which receptors can be endogenous or recombinant.

hormones and other cellular factors, and may also be used in methods for screening compounds for hormone/cellular factor activity and to identify agents which function as agonists or antagonists.

According to various embodiments of the invention, recombinant MAP2 kinase molecules can be used to create novel model systems for the study of mechanisms of hormones and other cellular factors. For example, and not by way of limitation, the recombinant molecules of the invention can be incorporated into cells or organisms such that higher than normal amounts of MAP2 kinase are produced, so that the effects of hyperactivation of MAP2 kinase may be evaluated. Overproduction of MAP2 kinase may identify aspects of the hormonal/cellular factor response related to MAP2 kinase activity, particularly when evaluated in comparison to cells or organisms which produce normal amounts of MAP2 kinase.

Alternatively, recombinant MAP2 kinase molecules may be engineered such that cells or organisms comprising the recombination molecules produce a mutant form of MAP2 kinase which may, for example, lack the serine/threonine kinase activity of normal MAP2 kinase. The mutant kinase may, on a concentration basis, overshadow, or titrate out, the effects of normal MAP2 kinase and thereby create cells or organisms with a functional aberrancy of MAP2 kinase function. It is also envisioned that such mutant nucleic acid sequences may result in mutation of the endogenous MAP2 kinase gene, for example, by homologous recombination, creating true MAP2 kinase mutants. In light of the high levels of expression of MAP2 kinase encoding mRNA in the central nervous system, and the role of MAP2 in forming neurofibrillary tangles, it may be possible to generate a transgenic non-human animal which expresses a mutant MAP2 kinase molecule in its central nervous system (e.g. via a brain-specific promoter sequence) and which may serve as an

animal model system for neurological disorders such as Alzheimer's disease or for peripheral neuropathies.

In addition, because the present invention enables the production of large amounts of purified MAP2 kinase for the first time, it allows for the production of anti-MAP2 kinase antibodies. Anti-MAP2 kinase antibodies, polyclonal or monoclonal, may be used in experiments utilizing cells or organisms which study the effects of selective neutralization of MAP2 kinase function. Such experiments may further elucidate the specific role of MAP2 kinase in hormone or cellular factor action.

An important embodiment of the present invention relates to methods for the screening of compounds for hormone or cellular factor activity. In specific embodiments, the present invention provides for a method of detecting the presence of a compound having nerve growth factor-like activity comprising (i) culturing cells that produce an MAP2 protein kinase (which is activated by nerve growth factor) in the presence of a compound suspected of having nerve growth factor-like activity (construed to mean activity similar but not necessarily identical to NGF, including, for example, the ability to support the growth of sympathetic neurons in culture) and (ii) detecting changes in the levels of MAP2 protein kinase activity, wherein an increase in activity is indicative of the presence of nerve growth factor-like activity. Similarly, in another specific embodiment, the present invention provides for a method of detecting the presence of a compound having insulin-like activity comprising (i) culturing cells that produce an MAP2 protein kinase (which is activated by insulin) in the presence of a compound suspected of having insulin-like activity (construed to mean activity similar but not necessarily identical to insulin, including for example, the ability to activate MAP2 kinase in insulin, responsive cells) and (ii)

kinase. For the production of antibody, various host animals can be immunized by injection with MAP2 kinase protein, or fragment or derivative thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and, Corynebacterium parvum.

A molecular clone of an antibody to a MAP2 kinase epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

The present invention provides for antibody molecules as well as fragments of such antibody molecules.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

5.5. BIOASSAYS FOR MAP2 KINASE ACTIVITY

The activity of MAP2 kinase may be measured using any suitable kinase assay known in the art. For example, and not by way of limitation, the method described in (Boulton et al., 1990, J. Biol. Chem. 265:2713-2719) as follows. The assay for phosphorylation of MAP2 may contain 30 mM Hepes, pH 8, 50 μM ATP (1-50 cpm/fmol), 1 mM dithiothreitol, 1 mM benzamidine, 10 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 3 μg MAP2 and no more than about 10 μg sample protein in a final volume of 30μl for 10 minutes at 30°C. The amount of MAP2 in the assay (100 μg/ml) may be chosen for convenience of analysis both by SDS-PAGE and by precipitation. The enzyme is not saturated with substrate even at 1.36 mg MAP2/ml. However, with 100 μg MAP2/ml enzyme activity may be expected to be linear with time for at least 30 minutes. All samples except for unfractionated supernatants may be routinely assayed as above in the presence of 1 mg of bovine serum albumin. Assays may be terminated by the addition of 10% trichloroacetic acid and precipitates may be collected on glass fiber filters. All assays except as noted above may be terminated by the addition of 0.25 volume of 0.3 M Tris-HCl, pH 6.9, containing 2 M mercaptoethanol, 50% glycerol and 10% SDS and analyzed by electrophoresis in SDS using 5% (MAP2) polyacrylamide gels. The gels may be stained with Coomassie blue, destained in 10% methanol and 10% acetic acid, dried and subjected to autoradiography at -80°C using Kodak XS-5 or BB-5 film with Dupont Quanta III intensifying screens. Substrate bands may be excised from gels and ^{32}P may be quantitated using liquid scintillation counting.

5.6. UTILITY OF THE INVENTION

The present invention may be utilized to provide unique model systems for the study of mechanisms of